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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Arthur M. Brown
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For	:	METHOD OF INDUCING APOPTOSIS IN HYPERPROLIFERATIVE CELLS
Confirmation No.	:	1521
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Examiner	:	Anne Marie Falk
Attorney Docket No.	:	CWR-019285US CON-1

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

APPEAL BRIEF

Sir:

Pursuant to the Notice of Appeal filed with respect to the application identified above, Appellants present herewith their Brief on Appeal.

I. REAL PARTY IN INTEREST

The real parties in interest is, Case Western Reserve University as indicated by the assignment recorded Reel No: 021615/Frame: 0991

II. RELATED APPEAL AND INTERFERENCES

There are no related appeals, interferences, or judicial procedures under 37 C.F.R. §41.37(1)(c)(ii).

III. STATUS OF CLAIMS

Claims 1-3, 5-6, 9-14, 16-25 are cancelled.

Claims 4, 7, 8, 15 and 26-39 are rejected and are appealed.

IV. STATUS OF AMENDMENTS

There are no pending amendments.

V. SUMMARY OF THE CLAIMED SUBJECT MATTER

A first aspect of the present invention, which is recited in claim 4, is directed to a method for inducing apoptosis in human prostate cancer or breast cancer cells ([0011], [0047], [0115] and [0116]). The method includes delivering to and expressing in the cells a nucleic acid ([0068], [0100] and [0114]). The nucleic acid includes a nucleotide sequence encoding human KChAP protein ([0062] and [0093]) and a promoter active in the cancer cells ([0011] and [0062]). The promoter is operably linked to the sequence encoding the protein ([0011] and [0093]). The cancer cells are in a tumor in a subject ([0100] and [0114]-[0116]), and the nucleic acid is in a viral vector ([0063]-[0067] and [0093]) which is delivered to the cancer cells by intratumoral injection ([0074], [0080] and [0114]).

Claim 7, which depends from claim 4 recites that the cancer cells comprise a native p53 protein ([0012], [0061] and [0111]).

Claim 8, which depends from claim 4 recites that the cancer cells comprise a mutant p53 protein ([0012], [0061] and [0113]).

Claim 15, which depends from claim 4 recites that the nucleic acid encodes a protein having the sequence set forth in SEQ ID NO: 2 ([0028]).

Claim 26, which depends from claim 4 recites that the cancer cells are human prostate cancer cells ([0050] and [0116]).

Claim 27, which depends from claim 4 recites that the cancer cells are human breast cancer cells ([0050] and [0117]).

Claim 28, which depends from claim 4 recites that the KChAP protein is expressed in said cancer cells at an amount effective to increase the level of phosphorylation on serine 15 of p53 relative to a control level ([0020] and [0109]).

Claim 29, which depends from claim 4 recites that the KChAP protein is expressed in said cancer cells at an amount effective to decrease the level of cyclins A and B relative to a control level ([0022] and [0111]).

Claim 30, which depends from claim 4 recites that the KChAP protein is expressed in said cancer cells at an amount effective to increase the level of cyclin D3 relative to a control level ([0022] and [0111]).

Claim 31, which depends from claim 4 recites that the KChAP protein is expressed in said cancer cells at an amount effective to increase the level of p53 relative to a control level ([0020] and [0109]).

Claim 32, which depends from claim 4 recites that the KChAP protein is expressed in said cancer cells in an amount effective to increase the level of cleaved poly(ADP-ribose) polymerase (PARP) relative to a control level ([0018] and [0108]).

Claim 33, which depends from claim 4 recites that the KChAP protein is overexpressed in said cancer cells relative to a control level ([0023]-[0024], [0047] and [0108])

A second aspect of the present invention, which is recited in claim 34, is directed to a method of a method for inducing apoptosis in human prostate cancer or breast cancer cells ([0011], [0047], [0115] and [0116]). The method includes delivering to and expressing in the cells a nucleic acid ([0068], [0100] and [0114]). The nucleic acid includes a nucleotide sequence encoding human KChAP protein ([0062] and [0093]) and a promoter active in the cancer cells ([0011] and [0062]). The promoter is operably linked to the sequence encoding the protein ([0011] and [0093]). The cancer cells are in a tumor in a subject ([0100] and [0114]-[0116]), and the nucleic acid is in a viral vector ([0063]-[0067] and [0093]) which is delivered to the cancer cells by intratumoral injection ([0074], [0080] and [0114]). The nucleotide sequence is delivered to the cancer cells in an amount effective to cause overexpression of the human KChAP protein as compared to a control level ([0023]-[0024], [0047] and [0108]).

Claim 35, which depends from claim 34 recites that the KChAP protein is expressed in said cancer cells at an amount effective to increase the level of phosphorylation on serine 15 of p53 relative to a control level ([0020] and [0109]).

Claim 36, which depends from claim 34 recites that the KChAP protein is expressed in said cancer cells at an amount effective to decrease the level of cyclins A and B relative to a control level ([0022] and [0111]).

Claim 37, which depends from claim 34 recites that the KChAP protein is expressed in said cancer cells at an amount effective to increase the level of cyclin D3 relative to a control level ([0022] and [0111]).

Claim 38, which depends from claim 34 recites that the KChAP protein is expressed in said cancer cells at an amount effective to increase the level of p53 relative to a control level ([0020] and [0109]).

Claim 39, which depends from claim 4 recites that the KChAP protein is expressed in said cancer cells in an amount effective to increase the level of cleaved PARP relative to a control level ([0018] and [0108]).

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

Whether claims 4, 7, 8, 15 and 26-39 are enabled under 35 U.S.C. §112, first paragraph.

VII. ARGUMENTS FOR CLAIMS 4, 7, 8, 15, and 26-39 IN VIEW OF THE 35 U.S.C. §112, FIRST PARAGRAPH REJECTION

Claims 4, 7, 8, 15 and 26-39 stand rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for a method of inducing apoptosis in cultured cancer cell lines, comprising the step of introducing into said cells *in vitro* an expression vector comprising a nucleic acid encoding a human KChAP protein as set forth in SEQ ID NO: 2, said nucleic acid operably linked to a

promoter active in cancer cell lines, does not reasonably provide enablement for the full scope of the claims.

The most recent Office Action, dated September 14, 2010, argues that the claims broadly embrace a method of tumor cancer therapy and that the specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims.

The Office Action argues that Kerbel, *Cancer & Metastasis Rev.* 17:301-304, 1999 (Hereinafter, "Kerbel 1"), Vieweg *et al.*, *Cancer Investig.* 13(2):193-201, 1995 (hereinafter, "Vieweg"), and Hoffman, *Invest. New Drugs* 17: 343-360, 1999 (hereinafter, "Hoffman") demonstrate that orthotopically transplanted tumors do not necessarily recapitulate the 'encouraging' responses of their ectopically (usually subcutaneous) grown counterparts, and that the animal model exemplified in the instant specification, *i.e.*, subcutaneously-growing human cancer cell lines in immunodeficient mice, does not sufficiently represent clinical human cancer, especially with regard to metastasis and drug sensitivity .

The Office Action further argues that a cancer cell line is not comprised of a mixture of cancer cells and normal cells, and that overexpression of K^+ channels in a subject's cells would likely also produce deleterious undesired consequences with respect to the promotion of unwanted cell proliferation. The Office Action concludes that a person of skill in the art would need to carry out further experimentation, with an uncertain outcome and constituting undue experimentation to introduce a viral

expression vector encoding KChAP, to be effective in inducing apoptosis and or treating a subject with prostate or breast cancer *in vivo.*

Appellants traverse the foregoing rejection as applied to claims 4 and 34 and submit that the amount of direction or guidance disclosed in the specification is sufficient to enable the skilled artisan to make and use the claimed methods using only routine experimentation.

"[T]o be enabling, the specification...must teach those skilled in the art how to make and use *the full scope of the claimed invention* without 'undue experimentation.'" *Id.* at 1561 (emphasis added), *quoted in Genentech, Inc. V. Novo Nordisk A/S*, 108 F.3d 1361, 1365 (Fed. Cir. 1997). Thus, "there must be sufficient disclosure, either through illustrative examples or terminology, to teach those of ordinary skill how to make and use the invention as broadly as it is claimed." *In re Vaeck*, 947 F.2d 488, 496 & n. 23 (Fed. Cir. 1991), *quoted in Enzo Biochem, Inc. V. Calgene, Inc.*, 188 F.3d 1362, 1374 (Fed. Cir. 1999). Some experimentation, even a considerable amount, is not "undue" if, *e.g.*, it is merely routine, or if the specification provides a reasonable amount of guidance as to the direction in which the experimentation should proceed. *See In re Wands*, 858 F.2d 731, 737 (Fed. Cir. 1988).

Facts that should be considered in determining whether a specification is enabling include: (1) the quantity of experimentation necessary to practice the invention; (2) the amount of direction or guidance presented; (3) the presence or absence of working examples; (4) the nature of the invention; (5) the state of the prior art; (6) the relative skill of those in the art; (7) the predictability or

unpredictability of the art; and (8) the breadth of the claims. *In re Wands,* 858 F.2d 731, 737 (Fed. Cir. 1988).

Appellants respectfully put forth that the specification of the present application provides guidance and direction to the skilled artisan commensurate with the scope of the claims. The specification of the present application teaches that apoptosis can be induced in prostate cancer cells or breast cancer cells by delivering and expressing a nucleic acid encoding human KChAP protein (in a viral vector) via intratumoral injection. The specification notes that the present method is especially useful for treating a patient with an epithelial carcinoma, such as breast cancer or prostate cancer (¶0047). For example, the specification notes that apoptosis may be induced in cancer cells, particularly prostate cancer cells, by introducing a KChAP protein in the cell (¶0070). The specification also notes that polynucleotides comprising a coding sequence for KChAP protein can include a promoter that permits expression of the protein (¶0062). Additionally, the specification notes that viral vectors may be used to deliver the KChAP polynucleotide to the cell (¶0063). Further, the specification notes that delivery of the KChAP polynucleotide may be via intratumoral injection (¶0075).

The specification of the present application also includes several working examples demonstrating that delivery and expression of a nucleotide sequence encoding human KChAP protein induces apoptosis in human prostate cancer and breast cancer cells. Example 1 of the specification demonstrates that delivery and expression of a nucleotide sequence encoding KChAP induces apoptosis in LNCaP cells, which are a prostate cancer cell line containing native p53 protein. Example 2

of the specification demonstrates that delivery and expression of a nucleotide sequence encoding KChAP induces apoptosis in Du145 cells, which are cells from a prostate cancer cell line containing mutated p53 protein. Example 3 of the specification demonstrates that *in vivo* growth of subcutaneous implants of human prostate cancer cells is inhibited by increasing intracellular levels of KChAP. Example 4 of the specification demonstrates that delivery and expression of a nucleotide sequence encoding human KChAP protein induces apoptosis in mammary epithelial cancer cells (*i.e.*, MCF-7 cells). Taken together, Examples 1-4 demonstrate that delivery and expression of a nucleotide sequence encoding human KChAP protein induces apoptosis in both prostate cancer cells and breast cancer cells.

Additionally, in contrast to the Examiner's assertion, the animal models used in the present application do sufficiently represent clinical cancer to enable a skilled artisan to practice the invention. The final rejection under 35 U.S.C. 112, first paragraph, is predicated, in part, on a belief that the animal model exemplified in the instant specification, *i.e.* subcutaneously-growing human cancer cell lines in immunodeficient mice, is unpredictable on its face, and that the working examples embody a number of deficiencies that do not allow one of skill in the art to extrapolate their teachings to applications wherein a cancerous breast or prostate tumor may be treated in a human subject. The Examiner states: "The evidence of record as a whole indicates that the heterotopic subcutaneous xenotransplantation of cells lines in an immunodeficient nude mouse fails to reflect human carcinoma, and therefore a person of skill in the art would need to carry out further experimentation,

with an uncertain outcome and constituting undue experimentation to introduce a viral expression vector encoding KChAP, to be effective in inducing apoptosis and or treating a subject with prostate or breast cancer *in* vivo." (Office Action, dated 9/14/10, page 7).

The author of the Kerbel 1 article cited in the Office Action also authored a more recent article, Kerbel, Cancer Biology & Therapy, 2:4: Suppl., S134-S139, 2003 (hereinafter, "Kerbel 2"), that discusses: 1) the pros and cons of different animal models of human carcinoma; 2) specifically that the xenograft animal model used to support the claimed invention is sufficient to reflect human carcinoma; and 3) that the alternate models proclaimed in the most recent Office Action as preferred models to the xenografts model of the present specification have drawbacks.

Kerbel 2 states: "Human tumor xenografts – even non metastatic ectopic/subcutaneous 'primary' tumor transplants – can be remarkably predictive of cytotoxic chemotherapeutic drugs that have activity in humans, when the drugs are tested in mice using pharmacokinetically clinical equivalent or 'rational' drug doses" (Kerbel 2, p.S134). Kerbel 2 further states that the skepticism levied at human tumor xenograft models based on the disparity between some results seen in mice versus result from clinical trial is not always warranted because "a crucial and fundamental difference between virtually all published experimental mouse therapy studies and corresponding clinical trials" is that "in most phase I, II and II clinical trials the patients being treated have advanced, high-volume metastatic disease whereas most mouse studies do not test the effects of therapy on advanced metastatic disease, but rather on primary tumor transplant or spontaneously rising primary

tumor, or microscopic, low-volume metastatic disease" (p. S137). In addition, Kerbel 2 states that "one of the limitations of many of the current transgenic oncomouse models" is that "they usually do not spontaneously metastasize" (p.S139). "The wisdom of the rush towards exclusive use of much more expensive transgenic oncomouse models for drug therapy testing *can be questioned*, especially when such tumors fail to express the most critical element of malignant disease – ability to metastasize, and the fact that less expensive transplantable tumor models are available which work – if used appropriately" (Kerbel 2, p139, emphasis added). Therefore, Appellants respectfully submit that, in view of Kerbel 2 and the inherent limitations present in all murine models of human carcinoma, the animal model used to support the claimed invention is sufficient to reflect human carcinoma.

Thus, it appears that the Examiner is rejecting these claims, based in part, on a bias against ectopic subcutaneous (s.c.) xenotransplantation of cancer cell lines in an immunodeficient nude mouse. However, s.c. xenotransplantation of cancer cell lines in an immunodeficient nude mouse is a widely-accepted experimental animal model of human carcinoma which is recognized by the relevant scientific community for pre-clinical screening of potential therapeutics. According to Kerbel 2, discussed above, human tumor xenografts used to support the claimed invention are sufficient to reflect human carcinoma. There is no valid reason why inventions supported by working examples including the use of s.c. xenotransplantation of cancer cell lines in an immunodeficient nude mouse should undergo a different or stricter standard of enablement than any other therapy.

The most recent Office Action also states that, "the working examples cited by Applicants are not commensurate in scope with the claimed invention and are directed to using cell lines, that in turn fail to reflect the issues raised for normal breast and prostate tumors, comprising a mixture of normal and transformed cells." As discussed above, according to Kerbel 2, the animal model described in the present specification by the Appellant are clinically relevant. Further, in contrast to the Examiner's position, the specification also teaches that KChAP induces apoptosis in both p53 wild-type and p53 mutant cells, thereby demonstrating efficacy in heterogeneous cancer cell types. Furthermore, the breadth of a claim is not in itself a reason justifying rejection of the claims for lack of enablement. "The first paragraph of §112 requires nothing more than objective enablement...How such a teaching is set forth, either by the use of illustrative examples or by broad terminology, is irrelevant." In re Vaeck, 947 F.2d 488, 496 n.23, 20 USPQ2d 1438, 1445 n.23 (Fed. Cir. 1991). The proper inquiry is whether a person of skill in the art could make and use the invention based on the specification without undue experimentation. Therefore, Appellants respectfully submit that the examples are sufficiently commensurate in scope with the claimed invention and, therefore, do enable the skilled artisan to make and use the method in the claims using only routine experimentation.

Moreover to ascertain the "level of predictability" in the context of a specific claimed invention, one must consider the specific elements of the invention, the predictability of success of each element and the ultimate predictability of success of the method as a whole. See *In re Marzocchi,* 439 F.2d 220, 169 U.S.P.Q. 367

(CCPA 1971); MPEP §2164.03. It is true that, in some instances, the unpredictable nature of the art "alone be enough to create a reasonable doubt as to the accuracy of a particular broad statement". *Marzocchi,* 169 U.S.P.Q. at 369-370. However, where "the effect of a change within the subject matter to which the claimed invention pertains" is reasonably predictable, enablement can be found. Here, and for the reasons set forth in more detail below, the Examiner has failed to meet the burden of establishing unpredictability which precludes enablement absent undue experimentation, *Marzocchi,* 169 U.S.P.Q. at 370.

Additionally, the Examiner's cited references do not establish unpredictability in the relevant art. The Examiner states that "the evidence of record as a whole indicates that the heterotopic subcutaneous xenotransplantation of cell lines in an immunodeficient nude mouse fails to reflect human carcinoma, and therefore a person of skill in the art would need to carry out further experimentation, with an uncertain outcome and constituting undue experimentation to introduce a viral expression vector encoding KChAP, to be effective in inducing apoptosis and or treating a subject with prostate or breast cancer *in vivo*." (page 7, Office Action mailed 9/14/10). To support this broad statement, the Examiner cites references to establish that, at the time of filing, "orthotopically transplanted tumors do not necessarily recapitulate the 'encouraging' responses of their ectopically (usually subcutaneous) grown counterparts, and that the animal model exemplified in the instant specification, i.e. subcutaneously-growing human cancer cell lines in immunodeficient mice, do not sufficiently represent clinical cancer, especially with regard to metastasis and drug sensitivity." (page 4-5, Office Action, mailed 9/14/10).

Per the standard for determining enablement under 35 U.S.C. § 112, it is not necessary for Appellants to demonstrate that success was "routine" or that there was sufficient experimentation for "general" success to be achieved. If the specification adequately teaches one of skill in the art to make and use the claimed invention without undue experimentation, regardless of whether there is "routine" or "general" success in the art, then the invention is enabled. Moreover, MPEP § 2164.03 provides that "[t]he 'predictability' or a lack thereof in the art refers to the ability of one skilled in the art to extrapolate the disclosed or known results to *the claimed invention."* (emphasis added). Therefore, what is relevant is not predictability in the general field of human carcinoma murine xenotransplantation models and their correlation to clinical success, but rather, the predictability of inducing apoptosis in prostate and breast cancer cells as it relates to the claimed subject matter, specifically the delivery and expression of a nucleotide sequence encoding human KChAP protein and a promoter to prostate and breast cancer cells *in vivo* for the claimed effects. Furthermore, the context of "general" success must be placed properly. The PTO is not concerned with the general success of inducing apoptosis of cancer cells in the marketplace but the general success in the context of obtaining a pharmacologically relevant effect. See Brana, 34 U.S.P.Q.2d at 1442-43.

When taken in context, the references cited in the Office Action do not support Examiner's contentions that subcutaneously-growing human cancer cell lines in immunodeficient mice to reflect human carcinoma was unpredictable in the context of enablement at the time of filing. In fact, these references not only fail to establish that animal model exemplified in the instant specification was regarded as unreliable,

they actually support the Appellants position that the expression of KChAP in prostate and breast cancer cells do yield predictable results. The most recent Office Action references Wang, *Eur. J. Physiol.* 448:274-286, 2004 (hereinafter, "Wang") and Shieh *et al.*, Pharmacol. Rev. 52:557-93, 2000 (hereinafter, "Shieh"), to support the Examiner's assertion of the unpredictability with regard to K+ channel expression. The Office Action maintains the pending claims are not enabled because of the "paradox that enhancement of K+ channel activity can facilitate not only tumor cell apoptosis but also tumor cell proliferation, especially in a tumor mass comprising a mixed cell population, bringing into question the validity of the claimed method as a therapeutic." However, Appellants respectfully submit that the Examiner has improperly broadened the claimed invention to include the expression of all K+ channels in general. The claimed invention does not encompass expression of all K+ channels in general. Rather, the claimed invention is directed towards the expression of a specific protein – KChAP. As discussed in more detail below, KChAP boosts expression of a subset of K+ channels (not all K+ channels in general), and is a pro-apoptotic regulator of prostate and breast cancer cells, not a pro-ongogenic. Thus, the Examiner's assertion of unpredictability with regard to K+ channel expression is misplaced.

In contrast to the Examiner's position, the expression of KChAP is indeed predictable. First, the Office Action cites to Wang in support of its assertion of the unpredictability of the claimed invention. "K+ channels favor tumor cell proliferation; therefore, inhibition of K+ channel function or down-regulation of K+ channel expression should inhibit tumorigenesis. On the other hand, K+ channels also

promote apoptotic cell death enhancement of K+ channel activity can facilitate not only tumor cell apoptosis but also tumor cell proliferation. This apparent paradox confounds the manipulation of K+ channel function and/or expression as an option for the treatment of cancers." (Wang, pp.281-282 bridging).

On the contrary, Wang actually distinguishes KChAP function from the above general statement with regard to K+ channel function. "KChAP boosts protein expression of a *subset* of K+ channels" (Wang, p279, column 2, emphasis added). Therefore, Wang actually teaches that the function of KChAP is *specific* to a subset of K+ channels, indicating a more *specific* and *predictable* result as opposed to a broad spectrum K+ channel activator. Furthermore, Wang also states that "[c]onsistent with its pro-apoptotic properties, KChAP prevents the growth of DU145, another prostate cancer cell line, and LNCaP tumour xenografts in nude mice, indicating that infection with Ad/KChAP might represent a novel method of cancer treatment" (p279-80). Thus, Wang merely references KChAP as a *pro-apoptotic* regulator and makes no reference to KChAP being a *pro-oncogenic*. Therefore, the "paradox" presented by Wang is not present in the claimed invention because the claims specifically identify KChAP. Moreover, there has been no evidence presented by the Examiner that KChAP does induce apoptosis in pancreatic and breast cancer cells.

Second, to further support its assertion as to the unpredictability of the claimed invention, the Office Action cites Shieh as a review of the prior art with regard to K+ channels as potential therapeutic targets at the time of the invention. "[Shieh] describe KChAP as a chaperone protein, or auxullary (*sic*) factor, regulating

the function and expression of the Kv Channels, such as Kv2.1, Kv1.3 and Kv4.3, and state that given the diversity of K+ channel subunits, understanding the composition of channel complexes *in vivo* remains a challenge" (Office Action p. 4).

However, this statement suggests that KChAP is known to bind to more Kv proteins than Kv2.1, Kv1.3, and Kv4.3, which is simply not true. Kuryshev, *et al.*, Am J Physiol Cell Physiol, 278: C931-41, 2000 (hereinafter, "Kuryshev"), is referenced by Shieh in support of the above quotation. Kuryshev demonstrate that, in a test for KChAP/Kv channel interactions, Kv2.1, Kv1.3, and Kv4.3 were the *only* proteins found to interact with KChAP out of 11 tested Kv channel proteins (Kuryshev, Fig. 2.). "Our results demonstrate that KChAP modulates the functional expression of *specific* Kv channels" (Kuryshev, p.C939, column 2, emphasis added). Therefore, Kuryshev indicates that KChAP interacts *specifically* and *predictably* with three Kv channel proteins.

Furthermore, the statement used in the Office Action with reference to Shieh "understanding the composition of channel complexes in vivo remains a challenge" (Shieh, p566, column 1), is used out of context and does not actually support the Office Action's assertion as to the unpredictability of the claimed invention. Shieh makes this statement in a larger discussion regarding combinations of all K+ channels in all tissue types (*Id.)* stating, "[g]iven the diversity of K+ channel subunits and the potential to vary the constituents to form diverse α- α or α-β heteromeric channel complexes to alter expression, cellular targeting, and biophysical and pharmacological properties in native cell types, understanding the precise composition of channel complexes in vivo remains a challenge". Appellants,

however, claim expressing KChAP to induce apoptosis in two subsets of cancer, breast and prostate, not the activation of all K+ channels found on all cells. Therefore, understanding the composition of KChAP channel complexes is not a challenge given their specificity and predictability as discussed above by Kuryshev Fig.2.

Next, the Examiner has quoted Shieh in support of the rejection of the claims: "K+ channel activities play important roles in signal transduction pathways leading to proliferation, differentiation and cell fusion, that enhancement of current is directly involved in apoptosis and oncogenesis, and that overexpression of rEAG K+ channels in Chinese hamster ovary or NIH 3T3 cells induces significant features characteristic of malignant transformation." However, Appellants fail to see the relevance of these statements because, in discussing K+ induced proliferation, Shieh does not make any specific references to KChAP or the three specific K+ channel proteins with which KChAP was known to interact (with reference to Kuryshev, Fig 2). In addition, the Examiner has failed to cite a reference to suggest that rEAG interacts with KChAP. Furthermore, neither Chinese hamster ovary cells nor NIH 3T3 cells are breast cancer cells or prostate cancer cells.

The Examiner has concluded that "the prior art of Shieh *et al.* highlights the unpredictability with regard to targeting K+ channel expression, and together with the post-filing art of Wang *et al.* argue against the overexpression of K+ channels, as such is known to promote malignancy." As discussed before, Appellants suggest that the Examiner has improperly broadened the claimed invention to include the overexpression or activation of all K+ channels. Appellants respectfully submit that

the claimed invention does not claim the overexpression of K+ channels generally, but instead claims the expression of KChAP (Claims 4 and 34, "...delivering to and expressing in said cells a nucleic acid comprising: i) a nucleotide sequence encoding human KChAP protein..."). In addition, as discussed above, because Kureyshev teaches that KChAP specifically interacts with Kv2.1, Kv1.3, and Kv4.3, the activity of KChAP is *predictable*. Therefore, the expression of KChAP in prostate and breast cancer cells do yield predictable results.

As noted above, the Examiner's rejection is based, in part, on a belief that the use of heterotropic s.c. xenotransplantation of human cancer cells lines in an immunodeficient nude mouse fails to reflect human carcinoma and is therefore unpredictable. To support this contention, the Examiner asserts that the use of s.c. xenotransplantation of cancer cell lines in working examples has many concerns:

> "The prior art teachings of Kerbel et al. (1999), Vieweg et al. (1995), and Hoffman et al. (1999) were cited to demonstrate that orthotopically transplanted tumors do not necessarily recapitulate the 'encouraging' responses of their ectopically (usually subcutaneous) grown counterparts, and that the animal model exemplified in the instant specification, i.e. subcutaneously-growing human cancer cells lines in immunodeficient mice, do not sufficiently represent clinical cancer, especially with regard to metastasis and drug sensitivity." (page 4-5, Office Action, mailed 9/14/10)

However, as noted above, the instant claims should not be subjected to a stricter standard of enablement simply because they are supported by working examples featuring s.c. xenotransplantation of cancer cell lines in immunodeficient nude ice.

Furthermore, the Examiner's opinion about the unpredictability of subcutaneously-growing human cancer cells lines in immunodeficient mice as a representation of clinical cancer is not supported by the art. The prior art, including references cited herein, clearly belies the Examiner's contentions that those skilled in the art viewed subcutaneously-growing human cancer cells lines in immunodeficient mice as a reflection of human carcinoma with a suspicion that it was unduly unpredictable. The skilled artisan with the specification's teachings before him or her, would reasonably expect that the delivery to and expression in human prostate cancer or breast cancer cells of a nucleotide sequence encoding human KChAP protein and a promoter as recited in claims 4 and 34, would obtain sufficient expression of the protein to produce the claimed results.

The requirements for FDA approval are in fact *more* stringent than requirements for enablement. For example, in *Scott v. Finney,* the Federal Circuit noted that "[t]esting for the full safety and effectiveness of a prosthetic device is more properly left to the Food and Drug Administration (FDA)." 34 F.3d 1058, 1063,32 U.S.P.Q.2d 1115, 1120 (Fed. Cir. 1994) (Exhibit 15). The *Scott* decision also cites a case which held that Congress has given the responsibility to the FDA, not the PTO, to determine whether drugs are sufficiently safe for human administration. While it is true that the present invention has not entered clinical trials, the fact that the animal model employed in the working examples of the invention is used in the bulk of current preclinical efficacy studies clearly establishes that human tumor xenograft models are not *per se* unpredictable and unbelievable. As stated in Hollingshead:

> "[t]he most common step following in vitro assays is efficacy assessments in rodent tumor models. Early in the history of cancer therapy development, a

> large variety of rodent tumors were used in efficacy assessments. However, in the early 1980's, immunologically compromised mice that are capable of supporting human tumor growth became more widely available. The availability of these mice resulted in the development of human tumor xenograft models, which are used in the bulk of current preclinical efficacy studies. Detractors from this approach have suggested that preclinical rodent-based tumor models are not predictive of human clinical outcomes and are therefore unnecessary and can be eliminated. However, it is important to note that although some drugs that show activity against human tumor xenografts have failed to show activity in human clinical trials, many of the clinically approved drugs in use today have demonstrated and continue to demonstrate activity in a variety of preclinical models." (Hollingshead, J Natl Cancer Inst 2008;100:1500-1510, at 1500).

Moreover, the Office Action's apparent position that the specification cannot teach how to use the claimed method unless it teaches solutions to all the problems in the field of cancer therapy is contrary to controlling case law. *See, e.g., In re Brana,* 51 F.3d 1560, 1568 (Fed. Cir. 1995).

In *Brana,* the claims were directed to compounds disclosed as anticancer agents. *Id.* at 1562. The USPTO rejected the claims as non-enabled, *id.* at 1563-64, despite working examples in Brana's specification showing treatment of cancer in a mouse model. *Id.* at 1562-63. The USPTO argued that the results of the mouse testing "are not reasonably predictive of the success of the claimed compounds for treating cancer in humans." *Id.* at 1567. The court concluded that this position "confuses the requirements under the law for obtaining a patent with the requirements for obtaining government approval to market a particular drug for human consumption." *Id.* The *Brana* court held that "[u]sefulness in patent law, and in particular in the context of pharmaceutical inventions, necessarily includes the expectation of further research and development. The stage at which an invention in

this field becomes useful is well before it is ready to be administered to humans." *Id* at 1568.

Here, the claims are directed to a method of inducing apoptosis in human prostate cancer or breast cancer cells, and Appellants' specification provides several working examples demonstrating just that both *in vivo* and *in vitro*. The Examiner has discounted the specification's working examples because "the animal model exemplified in the instant specification, i.e. subcutaneously-growing human cell lines in immunodeficient mice, do[es] not sufficiently represent clinical cancer, especially with regard to metastasis and drug sensitivity". However, Kerbel 2, teaches that xenografts mouse models are acceptable clinical cancer treatment models that yield results that can be indicative of clinical efficacy. Moreover, Appellants respectfully put forth that it is clear that those of skill in the art believed that the use of subcutaneously-growing human cancer cells lines in immunodeficient mice as a reflection of human carcinoma was reasonably predictable, particularly in the context of establishing enablement under 35 U.S.C. 112, first paragraph. Furthermore, enablement includes an expectation of further research and development. Thus, enablement is not precluded even if the claims encompass methods, such as prostate and breast cancer therapy that have not yet overcome all the obstacles to their clinical use. In sum, the fact that additional data is desirable to unambiguously confirm the observed results of the working examples, and that improvements are desirable, or even necessary, to commercialize the technology does not support an enablement rejection.

Accordingly, in contrast to the Examiner's assertions, the instant specification does enable one skilled in the art to practice the claimed invention and it would not require undue experimentation to practice the *claimed* method; specifically, a method of inducing apoptosis in human prostate cancer or breast cancer cells. Moreover, the reference relied upon by the Examiner provides no evidence that the claimed invention could not be successfully practiced. Appellants, therefore respectively submit that claims 4and 34 are enabled by the present application, and request that the 35 U.S.C. §112, first paragraph, rejection of these claims be withdrawn.

Claims 6-8, 15, 26-33 and 35-39 depend directly from claims 4 and 34, therefore Appellants respectfully request that the 35 U.S.C. §112, first paragraph, rejection of claims 6-8, 15, 26-27, 28-33, 35-39 also be withdrawn.

VIII. Conclusion

In view of the foregoing remarks, Appellants submit that the rejection of claims 4, 7, 8, 15 and 26-39 under 35 U.S.C. §112, first paragraph is improper. Appellants respectfully request that the rejection of the claims be withdrawn and the subject application be passed to issue.

Please charge any deficiency or credit any overpayment in the fees for this Appeal Brief to Deposit Account No. 20-0090.

Respectfully submitted,

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IX. CLAIMS APPENDIX

Claims 1-3 (Cancelled)

Claim 4 (Finally Rejected): A method for inducing apoptosis in human prostate cancer or breast cancer cells comprising:

delivering to and expressing in said cells a nucleic acid comprising:

i) a nucleotide sequence encoding human KChAP protein; and

ii) a promoter active in said cancer cells, wherein the promoter is operably linked to the sequence encoding said protein, wherein said cancer cells are in a tumor in a subject, and wherein said nucleic acid is in a viral vector which is delivered to said cancer cells by intratumoral injection.

Claims 5-6 (Cancelled)

Claim 7 (Finally Rejected): The method of claim 4 wherein the cancer cells comprise a native p53 protein.

Claim 8 (Finally Rejected): The method of claim 4 wherein the cancer cells comprise a mutant p53 protein.

Claims 9-14 (Cancelled)

Claim 15 (Finally Rejected): The method of claim 4, wherein the nucleic acid encodes a protein having the sequence set forth in SEQ ID NO: 2.

Claims 16-25 (Cancelled)

Claim 26 (Finally Rejected): The method of claim 4, wherein said cancer cells are human prostate cancer cells.

Claim 27 (Finally Rejected): The method of claim 4, wherein said cancer cells are human breast cancer cells.

Claim 28 (Finally Rejected): The method of claim 4, wherein said KChAP protein is expressed in said cancer cells at an amount effective to increase the level of phosphorylation on serine 15 of p53 relative to a control level.

Claim 29 (Finally Rejected): The method of claim 4, wherein said KChAP protein is expressed in said cancer cells at an amount effective to decrease the level of cyclins A and B relative to a control level.

Claim 30 (Finally Rejected): The method of claim 4, wherein said KChAP protein is expressed in said cancer cells at an amount effective to increase the level of cyclin D3 relative to a control level.

Claim 31 (Finally Rejected): The method of claim 4, wherein said KChAP protein is expressed in said cancer cells at an amount effective to increase the level of p53 relative to a control level.

Claim 32 (Finally Rejected): The method of claim 4, wherein said KChAP protein is expressed in said cancer cells in an amount effective to increase the level of cleaved poly(ADP-ribose) polymerase (PARP) relative to a control level.

Claim 33 (Finally Rejected): The method of claim 4, wherein said KChAP protein is overexpressed in said cancer cells relative to a control level.

Claim 34 (Finally Rejected): A method for inducing apoptosis in human prostate cancer or breast cancer cells comprising:

delivering to and expressing in said cells a nucleic acid comprising:

i) a nucleotide sequence encoding human KChAP protein; and

ii) a promoter active in said cancer cells, wherein the promoter is operably linked to the sequence encoding said protein, wherein said cancer cells are in a tumor in a subject, wherein said nucleic acid is in a viral vector which is delivered to said cancer cells by intratumoral injection, and wherein said nucleotide sequence is delivered to said cancer cells in an amount effective to cause overexpression of said human KChAP protein as compared to a control level.

Claim 35 (Finally Rejected): The method of claim 34, wherein said KChAP protein is expressed in said cancer cells at an amount effective to increase the level of phosphorylation on serine 15 of p53 relative to a control level.

Claim 36 (Finally Rejected): The method of claim 34, wherein said KChAP protein is expressed in said cancer cells at an amount effective to decrease the level of cyclins A and B relative to a control level.

Claim 37 (Finally Rejected): The method of claim 34, wherein said KChAP protein is expressed in said cancer cells at an amount effective to increase the level of cyclin D3 relative to a control level.

Claim 38 (Finally Rejected): The method of claim 34, wherein said KChAP protein is expressed in said cancer cells at an amount effective to increase the level of p53 relative to a control level.

Claim 39 (Finally Rejected): The method of claim 34, wherein said KChAP protein is expressed in said cancer cells in an amount effective to increase the level of cleaved PARP relative to a control level.

X. EVIDENCE APPENDIX

Hollingshead, Melinda G., "Antitumor Efficacy Testing in Rodents", J Natl Cancer Inst; Vol. 100, Issue 21, November 5, 2008, pgs. 1500-1510.

Kerbel, Robert S., "Human Tumor Xenografts as Predictive Preclinical Models for Anticancer Drug Activity in Humans", Cancer Biology & Therapy 2:4:Suppl. 1, S134-S139; July/August 2003.

Kuryshev, Yuri A., *et al.*, "KChAP as a Chaperone for Specific K+ Channels", Am J Physiol Cell Physiol, 278: C931-41, 2000

XI. RELATED PROCEEDING APPENDIX

There are no related appeals, interferences, or judicial procedures under 37 C.F.R. §41.37(1)(c)(ii).

APPENDIX

Antitumor Efficacy Testing in Rodents

Melinda G. Hollingshead

The preclinical research and human clinical trials necessary for developing anticancer therapeutics are costly. One contributor to these costs is preclinical rodent efficacy studies, which, in addition to the costs associated with conducting them, often guide the selection of agents for clinical development. If inappropriate or inaccurate recommendations are made on the basis of these preclinical studies, then additional costs are incurred. In this commentary, I discuss the issues associated with preclinical rodent efficacy studies. These include the identification of proper preclinical efficacy models, the selection of appropriate experimental endpoints, and the correct statistical evaluation of the resulting data. I also describe important experimental design considerations, such as selecting the drug vehicle, optimizing the therapeutic treatment plan, properly powering the experiment by defining appropriate numbers of replicates in each treatment arm, and proper randomization. Improved preclinical selection criteria can aid in reducing unnecessary human studies, thus reducing the overall costs of anticancer drug development.

J Natl Cancer Inst 2008;100:1500–1510

With the worldwide cancer death toll being reported as 7.6 million people in 2007 and current projections suggesting that nearly 1.5 million people living in the United States will be diagnosed with cancer in 2008 (1), there is an obvious need to develop more effective anticancer agents. Unfortunately, the costs of extensive preclinical research and development as well as those associated with generating the human clinical data necessary to support new agent approvals are extremely high. The drug development process includes many steps and requires substantial investments in time and resources, and ultimately requires the recruitment of patients who are willing to participate in human clinical trials (2). A 2003 estimate suggested that a single phase 3 clinical trial of anticancer chemotherapy involving 20 patients requires approximately 4000 person-hours of professional and technical time (3). In addition, the total research and development costs associated with a single compound are estimated to exceed $400 million (4). One contribution to these costs is the expense associated with initial development of compounds that are subsequently abandoned during the drug development process (4).

The typical development plan for a cancer chemotherapy agent involves sequential steps, each of which has associated costs that generally increase as the agent moves down the development path (2,3,5). These steps include in vitro studies to identify test agents; rodent studies to assess the potential activity of these agents; pharmacology studies to define drug absorption, distribution, metabolism, and elimination; and toxicology studies to define a safe starting dose for humans (2). The greatest costs are associated with the preclinical toxicology and pharmacology studies that are required before a drug can be tested in humans. Therefore, the earlier in the process that a compound is deemed unworthy of further development and dropped from consideration, the lower the costs will be for that agent and, ultimately, the lower the overall costs will be for new agents in general.

Although many different in vitro assays, both cell-based and molecular-target driven, have been used to identify lead compounds, the most common step following in vitro assays is efficacy assessments in rodent tumor models (6). Early in the history of cancer therapy development, a large variety of rodent tumors were used in efficacy assessments (7–11). However, in the early 1980s, immunologically compromised mice that are capable of supporting human tumor growth became more widely available (12,13). The availability of these mice resulted in the development of human tumor xenograft models, which are used in the bulk of current preclinical efficacy studies (6,8,11,14,15). Detractors from this approach have suggested that preclinical rodent-based tumor models are not predictive of human clinical outcomes and are therefore unnecessary and can be eliminated (6). However, it is important to note that although some drugs that show activity against human tumor xenografts have failed to show activity in human clinical trials, many of the clinically approved drugs in use today have demonstrated and continue to demonstrate activity in a variety of preclinical models (6,16–23). Because the selection of agents for advancement to human clinical trials has been and continues to be based, in part, on in vivo efficacy studies, their design and interpretation are important both ethically and economically.

In this commentary, I review factors that should be considered during preclinical drug testing to reduce the potential for false-positive conclusions while minimizing the risks of false-negative results. These factors include selecting an optimal preclinical efficacy model, developing a good experimental design, selecting a proper treatment plan, designing an experiment that will provide statistically valuable data, and, finally, presenting the data in a useful format to the research community. It is not my intent to present the pros and cons of the available models (eg, authochthonous vs xenograft, xenograft vs transgenic) because these issues

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See "Notes" following "References."

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have been discussed extensively elsewhere (6,10,24,25). For clarity, autochthonous models are those in which a tumor of mouse origin is transplanted into a mouse, whereas xenograft models involve the transplantation of a tumor from a heterologous species (eg, human) into a mouse. Transgenic models, generated by genetically altering the mouse genome to increase tumor occurrence, are also used in drug studies. Each of these models has unique features; however, the issues discussed here are applicable to all of them.

Identification of an Appropriate Species for Assessing Efficacy

One important consideration in testing new drugs in animal models is the identification of an appropriate species in which to conduct tumor studies, that is, one in which the compound will be effective. In this context, it is important to remember that many compounds are effective across a wide range of species. Indeed, veterinary medicine applies many of the same therapeutic agents across a diverse range of species, and many of these agents (eg, antibiotics, anti-inflammatory agents, analgesics, and anticancer therapies) are clinically relevant in humans. For a variety of reasons, rodent-based models are the most commonly used models for preclinical efficacy testing. In fact, well over 100 clinically approved anticancer compounds are active in rodent tumor models (http://dtp.nci.nih.gov). Nevertheless, important examples exist in which a compound is effective in a rodent model but not in humans. Potential reasons for such discordant results include 1) differences in the pharmacological behavior of drugs in rodents and humans, 2) toxicological differences between rodents and humans, 3) different growth rates of experimental rodent tumors vs spontaneous human tumors, 4) different tumor burden present in experimental models vs humans, 5) differences in objective measurements of outcomes in preclinical models vs humans, and 6) failure to develop and apply stringent evaluation criteria in the preclinical efficacy models (2,6,10,14,17). Although several of these variables can be identified and resolved during pharmacological and toxicological assessments, it is important to design and interpret the results from preclinical models cautiously to avoid sending inappropriate agents down the development pathway. Because issues of pharmacology, toxicology, and interspecies variations in physiology have been discussed elsewhere (6,10,14,17,19,23,25–27), I focus here on the issues specifically associated with selection of a proper model for preclinical experiments.

Selection of a Proper Tumor Model

The first step in preclinical in vivo efficacy evaluations of a chemotherapeutic agent is the selection of a proper tumor model. Early cancer drug development paradigms used a panel of rodent tumor models that were broadly applied to all test agents, that is, model selection was not drug specific (7,8,10). A number of the classical anticancer agents were therefore developed without a full understanding of their mechanism; they were often found to be broadly active in many rapidly growing tumors because of their nonspecific cytotoxic activity (eg, alkylating agents). Many agents that are now under development, by contrast, have been designed to interfere with a specific molecular target or pathway and thus do not possess broad-spectrum cytotoxic or cytostatic characteristics (5,8,10,14). Therefore, to study these agents it is important to identify a model that is capable of responding to alterations in the target pathway. The selection of an appropriate tumor model is commonly based on in vitro sensitivity profiles of the test agent against a panel of target cells or tumors. Alternatively, expression profiling of a panel of human or rodent tumors or transgenic mice can be used to select a potentially sensitive model (14,28–32).

The importance of target (gene or protein) expression for finding antitumor activity can be demonstrated by comparing the effects of the antiestrogen agent tamoxifen on MDA-MB-361 human estrogen receptor (ER)–positive breast cancer xenografts with its effects on MDA-MB-435 ER-negative melanoma (32–34) xenografts. As shown in Figure 1, the growth of MDA-MB-435 xenograft tumors was not inhibited by treatment with tamoxifen, whereas tamoxifen caused marked growth inhibition of the MDA-MB-361 xenograft tumors. The first step in avoiding unnecessary expenditures, as this example shows, is the selection of an efficacy model that is appropriate for the purported mechanism of the test agent—in this case, an antiestrogen would be unlikely to have activity against a tumor that does not express the ER. In addition, for tumors generated from passaged cell lines, it is important to verify the presence of the relevant target in the growing tumors and not just in the cell lines from which they originated, because

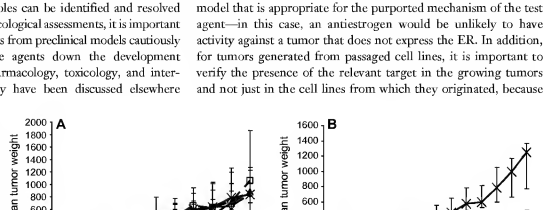

Figure 1. Activity of tamoxifen in human tumor xenografts in mice. **A)** MDA-MB-435 estrogen receptor–negative melanoma xenografts. **B)** MDA-MB-361 estrogen receptor–positive breast cancer xenografts. Cells of both lines were implanted orthotopically into the mammary fat pad of athymic nu/nu NCr mice (Animal Production Program, NCI-Frederick), and treatment was initiated when the tumors reached 150–175 mg in size. The MDA-MB-361 tumor-bearing mice were treated weekly with estradiol cypionate (20 μg per mouse) to support tumor growth. Exogenous estradiol is not required for progressive growth of MDA-MB-435 xenografts. For both studies, the vehicle control was 100% sesame oil given by oral gavage once daily for 20 days (n = 20 mice). Tamoxifen was administered by oral gavage once daily for 20 days at a dose of 45, 22.5, or 11.25 mg/kg (n = 10 mice per dose). Individual tumor weights were calculated as weight in mg = (length × width²)/2. Data are plotted as median tumor weight ± the 95% confidence interval of the median.

in vivo cultivation can alter target gene and protein expression through changes in environmental pressures.

During preclinical efficacy testing, prior knowledge of the sensitivity of the potential tumor models to clinically approved agents is also helpful. This information may strengthen the conclusion that activity in the model reflects the potential for activity in humans; more importantly, this information supports the expectation that the tumor can respond to chemotherapeutic intervention. For example, two clinically approved anticancer agents, temozolomide and topotecan, are effective against A375 melanoma xenograft tumors (temozolomide at a single dose of 400 mg/kg body weight or at three doses of 200 mg/kg, and topotecan at 1 mg/kg on a multidose schedule), whereas both agents are ineffective against human Colo 829 melanoma xenografts at the same doses (Figure 2). During the initial evaluation of a new anticancer agent, it is most productive to select a sensitive model (eg, A375 melanoma) for initial dose, route, and schedule studies and then to evaluate an optimized treatment protocol in more resistant models (eg, Colo 829 melanoma) as part of a sequential efficacy assessment paradigm.

Experimental Design Considerations

Selection of a sensitive model is an important first step in carrying out appropriate in vivo efficacy testing; however, developing a good experimental design is also of paramount importance because a poorly designed experiment results in a poorly supported conclusion (10). Important experimental design components include defining the proper controls, treatment protocols, group sizes, and randomization protocols. If these parameters are not considered, the efficacy of a new chemotherapeutic agent may be either over- or underestimated. It is also important to consider the potential impact of the compound formulation on experimental outcomes because of the possibility that the formulation itself may have direct effects on the tumor or the host (eg, unexpected toxic effects). Generally, test compounds are formulated in a carrier or diluent that is believed to be inert and is referred to as the drug vehicle. However, it is possible for the vehicle to be biologically active or to have an unexpected toxicity profile, particularly if new vehicle formulations are developed for the agent undergoing evaluation. Furthermore, experimental manipulation of animals induces a stress response that can alter the experimental outcome. Therefore, vehicle-treated animals should be used as experimental controls instead of untreated animals, even though doing so increases the total number of experimental animals that must be administered treatments during the experiments.

An example of how the selection of an appropriate vehicle and the inclusion of proper vehicle controls are critical to preventing misinterpretation is provided by data from a study in which a therapeutic agent directed against a target expressed in OVCAR-5 human ovarian tumor xenografts was evaluated for tumor growth inhibition (Figure 3). Because the therapeutic agent was found to have the best in vitro activity profile when it was prepared in a lipid-based vehicle, that vehicle was used for administering the drug in the in vivo study. In addition, the therapeutic agent was solubilized in phosphate-buffered saline (PBS) for comparison. In a Kaplan–Meier survival analysis (Figure 3), the lipid-based vehicle alone was as effective at improving survival as the therapeutic agent prepared in it. Furthermore, the vehicle alone was more effective than the therapeutic agent prepared in PBS. If this study had used only untreated or PBS-treated control animals, then the agent would have been assigned greater activity than it actually had. In addition to showing the importance of selecting an appropriate vehicle and including proper vehicle-treated control animals, this example shows the importance of providing the specific details of the control animals as part of the presentation of the experimental data, so that the reader can properly assess the experiment.

Development of a Treatment Plan

Selection of a model and the relevant controls should be straightforward; however, the options for a treatment protocol are limited only by the imagination of the operator. Several important factors should be considered when developing a treatment plan. The most important consideration is whether the experimental agent will be

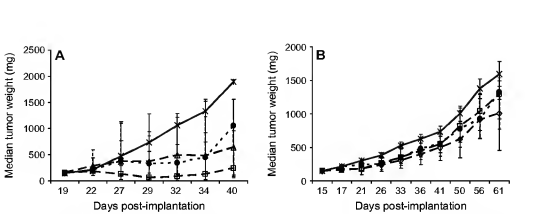

Figure 2. Activity of temozolomide and topotecan in human tumor xenografts. **A)** A375 melanoma xenografts. **B)** Colo 829 melanoma xenografts. Cells of both lines were implanted subcutaneously in female athymic nude (nu/nu NCr) mice (Animal Production Program, NCI-Frederick). Treatment was initiated when the tumors reached 150 mg. Temozolomide was administered by oral gavage as a single dose of 400 mg/kg or as three 200-mg/kg doses given 4 days apart (temozolomide 200 mg/kg × 3) (n = 10 mice per dose group). Topotecan was administered intraperitoneally at 1 mg/kg 5 days per week for 2 weeks (n = 10). The vehicle control group (n = 20) was treated with three doses of saline given 4 days apart. Individual tumor weights were calculated as weight in mg = (length × width²)/2. Data are plotted as median tumor weights ± 95% confidence interval.

physiologically available to the tumor. Bioavailability is affected by several factors, including the tumor growth site and vascularization of the tumor and surrounding tissues, the vehicle and treatment route, the solubility and stability of the test material, and the uptake, metabolic, and excretion pathways that affect the agent (6,10,14,17). A second critical consideration is the required therapeutic exposure; that is, what is the minimum exposure time required for the agent to affect the tumor cells, and is that effect reversible or irreversible? For example, if a minimum exposure of 48 hours is required to modulate the target, then a single dose of test agent will be unlikely to inhibit tumor growth unless the agent has a long in vivo half-life. Although biologic agents (eg, antibodies) often have long half-lives, most small molecules have short half-lives, particularly in rodents (5). Conversely, a test agent may have a rapid effect on the target, but if that effect is reversible, then antitumor activity will be lost rapidly unless repeated treatments are given.

During development of a treatment protocol, therefore, it is essential to give serious consideration to the likelihood that the tumor will have sufficient exposure to the test agent under the experimental conditions selected. Otherwise, a potentially valuable test agent may be discarded as ineffective during in vivo efficacy studies. A classic example is provided by the early work conducted with paclitaxel (35). In initial studies, paclitaxel was administered intraperitoneally as a suspension in saline rather than as a solution. Activity was modest in these early studies, and the compound was not actively pursued for several years, until subsequent studies using a different vehicle (cremophor, ethanol, and saline) and an intravenous route of administration revealed profound antitumor activity against a wide variety of tumor models (35). The dependence of paclitaxel's activity on the route of administration was ultimately explained when pharmacology studies unequivocally demonstrated a failure of systemic distribution following intraperitoneal administration (36). It is sobering to consider the impact of losing a compound, such as paclitaxel, that has substantial antitumor activity because of poor experimental design or failure to appreciate the issues associated with its particular biology.

Treatment protocols must have a clear basis for selecting doses and schedules for test agent administration. The goal may be 1) to achieve a target plasma concentration, 2) to maintain a minimum exposure time, or 3) to administer the maximum amount of test agent that does not cause unacceptable toxicity. Therapeutic protocols for anticancer drugs have often depended on the maximum tolerated dose to define the treatment dose and schedule (17). Although this approach may be successful with cytotoxic drugs, it may be less appropriate when assessing cytostatic or target-modulating agents (19,37). These agents are expected to require long-term continuous exposure of the tumor and would likely require similar dosing in humans; thus, administering them at a near-toxic dose is undesirable because it could lead to over-interpreting the activity of the compound, given that comparable doses would be unlikely in humans. Furthermore, target-modulating agents are expected to be of low toxicity because of their specificity. Thus, administration at the maximum tolerated dose would be expected to greatly exceed the necessary exposure, leading to unnecessary consumption of the test agent and thereby increasing the overall costs of testing. For agents with few toxic effects, treatment schedules may be developed using pharmacological endpoints such as plasma concentration and exposure time (19). Whether toxic effects or pharmacologic endpoints define the therapeutic doses and schedules, the conclusions drawn from a particular study are relevant only for the treatment conditions used in the assay. It is essential for the proper interpretation of in vivo therapeutic data that the treatment parameters be provided along with an explanation for their selection because this information is critical to a full understanding of the meaning of the experimental outcomes.

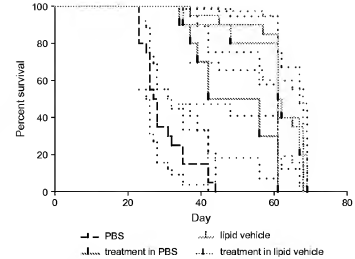

Figure 3. Kaplan–Meier analysis of survival of athymic nude mice bearing intraperitoneal OVCAR-5 human ovarian cancer xenografts. Mice were nu/nu NCr (Animal Production Program, NCI-Frederick). The therapeutic agent was administered at a dose of 1 mg per mouse given intraperitoneally once every other day, for a total of seven doses (n = 10 mice per group) using two different vehicles (lipid vehicle and phosphate-buffered saline [PBS]). The lipid vehicle (n = 20 mice) and PBS (n = 20 mice) were used in separate vehicle control groups following the same dosing schedule. Mice were treated with vehicle alone or with one of the therapeutic agents solubilized in the vehicles. **Dotted lines** indicate 95% confidence intervals.

Another consideration in the experimental design is the number of test animals per group. This is another critical feature of efficacy studies because too few animals can result in questionable or invalid results, whereas excessive numbers of animals add costs without producing commensurate benefit. Recommendations from a statistician about the minimum number of animals necessary to achieve appropriate statistical power during development of the experimental protocol can greatly improve the experimental design. If a statistician is not available, then a power calculator should be used to determine the minimum group sizes required to detect differences in measurable outcomes (eg, tumor size, life span) between groups. Such power calculations may be of particular benefit when designing experimental protocols for tumor models in transgenic mice, because these models are heterogeneous in their frequency and time of tumor occurrence. Finally, it is important to determine group sizes based on the efficacy model that is being used rather than relying on the replicate sample paradigm that is typical of in vitro assays, which generally results in groups of insufficient size.

For power calculations, one must know the expected mean values and SDs for the experimental endpoint that is being assessed

(eg, tumor size, survival time, target protein expression level). These values can be determined from historical data or from preliminary experiments. For example, growth data (M. G. Hollingshead, unpublished data) for subcutaneous MDA-MB-361 tumors grown from an inoculum of 1×10^7 cells in 0.1 mL given to each of six mice revealed tremendous variability in tumor growth among the mice (Table 1); these data suggest that the subcutaneous MDA-MB-361 tumor xenograft is not an ideal model for studying the therapeutic efficacy of antitumor agents. Using a commercially available power calculator (GraphPad StatMate 2, GraphPad Software, La Jolla, CA), the 95% confidence interval (CI) for the tumor growth data at day 68 (SD of 1401 mg) indicates that achieving a statistically significant difference in tumor growth between two groups would require a difference of 5246 mg if n = 3 mice per group, 3629 mg if n = 5 mice per group, 2945 mg if n = 7 mice per group, 2543 mg if n = 9 mice per group, and 2164 mg if n = 12 mice per group. By contrast, the same cell line inoculated into the mammary fat pad produced tumor growth data with much less variability (Table 2). In this instance, the SD in tumor growth at day 68 was 301 mg. The power calculation for this model indicates that statistically significant differences in tumor growth between the two groups would require a difference of 1127 mg if n = 3 mice per group, 780 mg if n = 5 mice per group, 633 mg if n = 7 mice per group, 546 mg if n = 9 mice per group, and 465 mg if n = 12 mice per group. Thus, intragroup tumor heterogeneity profoundly affects the group size necessary to determine the statistical significance of the difference in tumor growth and should be considered before a tumor model is selected and the experiment is designed. Due to the expense of including sufficient animals to conduct statistically powerful preclinical efficacy studies, many such studies include too few mice, yielding at best an experiment with minimum value and at worst, one with misleading conclusions. These underpowered experiments not only add to the final costs of drug development but also contribute data to reinforce the argument that preclinical models are not predictive of clinical outcomes.

In an ideal model, the tumor size distribution at any given observation time will be extremely small, so that subtle differences among groups will be easily detected and statistically significant. Unfortunately, rodent models, whether spontaneous (eg, transgenic) or transplanted, do not result in uniform tumor growth among all tumor-bearing animals. One way to reduce the impact of this variability is to create a large population of tumor-bearing mice and then select a homogeneous subset for randomization into the experimental groups. This approach, referred to as staging the tumor, allows the investigator to select a group of mice whose tumors are homogeneous so that differences in tumor size and age are initially minimized and distributed randomly to each treatment group.

Another often overlooked but important consideration in animal efficacy studies is proper randomization. If animals are assigned to groups on the basis of a biased selection factor rather than a randomization protocol, then the consequence will be experimental bias. For example, in tumor models in which the tumor is generated by implantation of tumor cells or tumor fragments, the inoculum is subject to time-dependent changes in viability, with

Table 1. Actual weights of MDA-MB-361 tumors grown subcutaneously in athymic nude (nu/nu Ncr) mice*

	Experimental day														
	5	8	11	15	19	26	29	33	36	40	43	47	54	61	68
Absolute weight, mg															
Mouse 1	14	63	63	63	63	63	63	63	63	75	75	148	211	307	325
Mouse 2	14	14	14	14	14	14	14	14	14	14	14	14	14	63	63
Mouse 3	14	14	14	14	14	14	14	14	63	63	63	81	144	253	425
Mouse 4	14	14	14	14	14	63	63	63	108	158	196	343	666	1276	1744
Mouse 5	14	63	63	63	63	63	81	106	144	343	496	817	1310	2588	3752
Mouse 6	14	14	14	14	14	14	14	14	14	63	63	81	208	425	600
Median tumor weight	14	14	14	14	14	38	39	39	63	69	69	115	210	366	513
Average tumor weight	14	30	30	30	30	38	42	46	68	119	151	247	425	819	1151
SD of average	0	25	25	25	25	27	31	38	51	119	180	301	486	964	1401
SD of median	0	31	31	31	31	27	31	39	52	131	201	334	541	1084	1566
t test vs day 68 values	.07	.08	.08	.08	.08	.08	.08	.08	.09	.10	.11	.15	.26	.64	NA
Relative weight†															
Mouse 1	1.0	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	5.4	5.4	10.6	15.1	21.9	23.2
Mouse 2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	4.5	4.5
Mouse 3	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	4.5	4.5	4.5	5.8	10.3	18.1	30.4
Mouse 4	1.0	1.0	1.0	1.0	1.0	4.5	4.5	4.5	7.7	11.3	14.0	24.5	47.5	91.1	124.6
Mouse 5	1.0	4.5	4.5	4.5	4.5	4.5	5.8	7.6	10.3	24.5	35.5	58.3	93.6	184.8	268.0
Mouse 6	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	4.5	4.5	5.8	14.9	30.4	42.9
Average	1.0	2.2	2.2	2.2	2.2	2.7	3.0	3.3	4.8	8.5	10.8	17.7	30.4	58.5	82.2
SD	0.0	1.8	1.8	1.8	1.8	1.9	2.2	2.7	3.7	8.5	12.8	21.5	34.7	68.8	100.1

* Tumor weights were calculated as tumor weight (mg) = (tumor length × tumor width^2)/2. Tumors that are shown as weighing 14 mg were estimated as being 3 × 3 mm because they were palpable but too small to be accurately measured with calipers. NA = not applicable.

† Relative tumor weight = (day *T* weight/day 5 weight), where day *T* is each time point beyond the day-5 starting mass.

the result that time to tumor occurrence, initial tumor size, and tumor growth rate will differ between animals implanted at the beginning of the procedure and those inoculated later in the process. If animals are assigned to groups on the basis of inoculation sequence (eg, first six mice to group 1, second six mice to group 2, and so on) instead of randomly, the result will be skewed outcomes. Operator fatigue is also a consideration, particularly with complex or difficult experimental designs (eg, those requiring surgical or intravenous tumor cell inoculation), and the impact of such operator-related bias should therefore be addressed by randomly assigning animals across the experimental groups. With transgenic mice that develop genetically induced tumors and with tumor models that are induced by exposure to chemical carcinogens (eg, skin tumor induction by painting with 7,12-dimethylbenz[*a*] anthracene and phorbol 12-myristate 13-acetate), there may be heterogeneity in the responses of different litters and age groups that should be considered during protocol development. The use of simple computer programs to randomize animals can avoid this experimental bias without burdening the investigator. The randomization method should be routinely reported as part of a properly described animal model experiment.

Choice of Endpoints

One goal of preclinical tumor models is to define the effect of an experimental treatment on the tumor. This goal requires the selection of an endpoint or set of endpoints. Endpoints should be defined in the experimental design, because selecting the endpoints based on experimental outcomes provides another opportunity to introduce bias. The two most common endpoint categories are antitumor activity and modulation of molecular targets.

Antitumor effectiveness can be defined in various ways, but the ultimate goal of any treatment is to decrease tumor burden, decrease tumor-associated morbidity, improve quality of life, and, where possible, lengthen life span, irrespective of the host species. To reproducibly measure efficacy, response to treatment must be assessed by a set of objective parameters (10,14,15,19,23). Human clinical responses can be defined in several ways. Obviously, the

Table 2. Actual weights of MDA-MB-361 tumors grown in the mammary fat pad of athymic nude (nu/nu Ncr) mice*

	Experimental day													
	13	19	22	26	29	33	36	39	46	50	53	56	60	63
Absolute weight, mg														
Mouse 1	108	117	148	208	196	196	272	343	466	625	756	893	1008	1367
Mouse 2	117	117	117	221	221	361	320	361	225	320	336	425	662	972
Mouse 3	158	225	253	325	288	385	385	550	696	726	756	1055	1172	1310
Mouse 4	108	144	225	361	320	338	416	676	972	1080	1172	1328	1044	1458
Mouse 5	75	98	117	196	225	288	325	429	550	575	600	787	1133	1268
Mouse 6	88	106	126	304	288	239	253	288	320	336	352	425	525	689
Mouse 7	98	117	126	239	225	325	343	405	726	787	893	1268	1458	1504
Mouse 8	125	125	180	211	245	245	336	336	425	486	564	756	847	992
Mouse 9	126	135	144	190	190	201	233	281	336	397	397	650	675	772
Mouse 10	144	144	196	405	405	446	486	564	650	847	926	1080	998	1269
Mouse 11	81	153	153	336	320	272	272	272	325	525	525	550	600	600
Mouse 12	135	272	272	425	361	474	662	725	625	787	817	1044	1172	1248
Mouse 13	81	117	117	225	208	208	208	253	384	662	600	600	756	893
Median	108	125	148	239	225	288	325	361	466	625	600	787	998	1248
Average	111	144	167	280	269	306	347	422	515	627	669	835	927	1105
SD	26	50	54	83	68	92	122	158	211	220	247	303	275	301
SD of the median	26	54	57	93	72	94	124	170	217	220	257	307	285	336
t test vs day-63 values	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	.0323	.129	NA
Relative weight†														
Mouse 1	1.0	1.1	1.4	1.9	1.8	1.8	2.5	3.2	4.3	5.8	7.0	8.3	9.3	12.7
Mouse 2	1.0	1.0	1.0	1.9	1.9	3.1	2.7	3.1	1.9	2.7	2.9	3.6	5.7	8.3
Mouse 3	1.0	1.4	1.6	2.1	1.8	2.4	2.4	3.5	4.4	4.6	4.8	6.7	7.4	8.3
Mouse 4	1.0	1.3	2.1	3.3	3.0	3.1	3.9	6.3	9.0	10.0	10.9	12.3	9.7	13.5
Mouse 5	1.0	1.3	1.6	2.6	3.0	3.8	4.3	5.7	7.3	7.7	8.0	10.5	15.1	16.9
Mouse 6	1.0	1.2	1.4	3.5	3.3	2.7	2.9	3.3	3.6	3.8	4.0	4.8	6.0	7.8
Mouse 7	1.0	1.2	1.3	2.4	2.3	3.3	3.5	4.1	7.4	8.0	9.1	12.9	14.9	15.3
Mouse 8	1.0	1.0	1.4	1.7	2.0	2.0	2.7	2.7	3.4	3.9	4.5	6.0	6.8	7.9
Mouse 9	1.0	1.1	1.1	1.5	1.5	1.6	1.8	2.2	2.7	3.2	3.2	5.2	5.4	6.1
Mouse 10	1.0	1.0	1.4	2.8	2.8	3.1	3.4	3.9	4.5	5.9	6.4	7.5	6.9	9.0
Mouse 11	1.0	1.9	1.9	4.1	4.0	3.4	3.4	3.4	4.0	6.5	6.5	6.8	7.4	7.4
Mouse 12	1.0	2.0	2.0	3.1	2.7	3.5	4.9	5.4	4.6	5.8	6.1	7.7	8.7	9.2
Mouse 13	1.0	1.4	1.4	2.8	2.6	2.6	2.6	3.1	4.7	8.2	7.4	7.4	9.3	11.0
Average	1.0	1.3	1.5	2.6	2.5	2.8	3.2	3.8	4.8	5.8	6.2	7.7	8.7	10.3
SD	0.0	0.3	0.3	0.8	0.7	0.7	0.8	1.2	2.0	2.2	2.3	2.8	3.1	3.3

* Tumor weights were calculated as tumor weight (mg) = (tumor length × tumor width²)/2. NA = not applicable.

† Relative tumor weight = (day *T* weight/day 13 weight), where day *T* is each time point beyond the day 13 starting mass.

primary goal of cancer therapy is to improve long-term survival while maintaining the patient's quality of life. However, this outcome is difficult to assess in the short term because patients represent a heterogeneous population with a heterogeneous collection of diseases. More commonly, therefore, clinical trials assess time to disease progression, objective response rates, surrogate markers, and quality of life parameters (38). Although not all of these outcomes translate directly to the preclinical models, they do offer an opportunity to define endpoints that may ultimately have clinical relevance (7,10,11).

A variety of endpoints for subcutaneous tumor models have been described in the literature and applied by pharmaceutical companies in their drug development pathway. Within the Developmental Therapeutics Program of the US National Cancer Institute (http://dtp.nci.nih.gov), endpoints for subcutaneous tumors include percent test/control (%T/C) tumor weights calculated on each day that tumors are measured, tumor growth delay, net log cell kill, median days to a defined tumor weight or to a specified number of tumor doublings, and tumor regression (7,11). The lowest calculated %T/C seen over time is defined as the optimal %T/C because it defines the greatest level of activity seen with the test agent. Many pharmaceutical companies use similar endpoints (10,20,39–41). The rate and duration of partial and complete tumor regressions are also considered to be clinically relevant endpoints (42,43), and tumor growth delay serves as a surrogate for disease progression.

The endpoints described here for subcutaneous tumors are calculated based on tumor mass as determined from caliper measurements of the length and width of the subcutaneous tumors. These measurements are subject to operator error and are commonly inaccurate for tumors smaller than 5 mm in either dimension, particularly on haired mice, because the thickness of mouse skin varies among mice and even across the surface of a single mouse and can variably affect caliper measurements. Because of the variability associated with caliper measurements of tumors smaller than 5×5 mm, tumor weights of less than 63 mg are unreliable and should be considered suspect when comparing tumor mass (11). In addition, because caliper measurements are operator dependent, it is important that the same operator measure the tumors throughout the course of the experiment. Finally, if the operator can be blinded to the experimental treatment group assignments, there is an even greater reduction in experimental bias.

For tumor models in which tumors grow in sites other than the subcutaneous compartment, an alternate means of tumor measurement must be identified before the therapeutic protocol is initiated. During protocol development, the accuracy and reproducibility of the endpoint measurements must be considered so that the data are properly collected and analyzed. For example, if tumors are to be resected and physically weighed, then the same criteria must be used for determining tumor borders in each mouse, irrespective of whether it was treated with vehicle or test agent. Another important variable is the accuracy of the balance used to weigh the tissues. To remove resection bias, tumors growing within organs can be assessed by resecting and weighing the entire organ. This approach should be used only if an adequate period of time has elapsed between tumor inoculation and measurement so that there is a sufficient tumor mass to provide statistically valid differences between treatment groups. Alternatively, visceral lesions can be assessed by histopathologic evaluation and manual or automated quantitation of the number or volume of lesions. In cases where multiple metastatic lesions occur, quantifying the number and size of lesions may provide an unbiased endpoint. The number of microscopic lesions is a common endpoint for models in which lung or liver metastases are present, such as the murine tumors Lewis lung, B16 melanoma, and M5076 sarcoma (39).

It is worth noting that new imaging technologies (eg, bioluminescence, ultrasound, magnetic resonance imaging) are providing improved, highly sensitive methods for assessing visceral tumor growth that may supplement many of the classical tumor assessments. These technologies also have limitations in that not all of them are equally valuable for all tumor growth sites and, in many cases, the endpoints have not been fully validated. For example, ultrasound can be used to assess tumors growing in the kidney but is not optimal for lesions in the lung (44). For various imaging endpoints, the operator defines the tumor margins when selecting the region of interest for measurement. As stated earlier, the importance of selecting a reproducible, unbiased endpoint is essential because the data generated may be used to make critical drug development decisions. Therefore, when using these technologies for assessing tumor growth, the methods used to define the tumor should be well characterized, reproducible, nonbiased, and validated.

All of these considerations are important to conducting nonbiased experiments; however, the value of the experiment to the research community depends on how the data are presented. Even with staged tumors, growth heterogeneity—particularly for human tumor xenografts—is unavoidable. This heterogeneity led the Developmental Therapeutics Program as well as many pharmaceutical companies to select the group median, rather than the group average, tumor weight for calculation of endpoints (7,8,10,11,20,40,41). The use of the median reduces the impact of an outlying tumor weight on the overall interpretation of the data. For most readers, it is easiest to understand tumor growth data if weights, whether graphed as medians or averages, are presented for each treatment group rather than as a calculated percent of control or starting tumor weight (ie, relative tumor weight). With graphs that show median or average tumor weights, the reader knows the actual tumor weights when treatment was initiated and terminated as well as the continued tumor behavior following cessation of treatment (15). By contrast, when tumor weight data are presented as relative weights, it is more difficult for an observer to assess the real impact of treatment on the tumor. For example, a tumor that is 200% of its starting weight could be 40, 200, or 2000 mg, depending on whether the starting tumor weight was 20, 100, or 1000 mg. Thus, when data are displayed as a relative tumor weight, the observer cannot determine whether the tumor is below the limit of accurate measurement for the assessment method employed. Furthermore, the true impact of treatment on the tumor is more obvious when median or average tumor weights, rather than calculated percentages, are presented visually, because the observer can determine the change in tumor size in terms of actual tumor weight.

Whether average or median tumor weights are presented, the SD, SE, or 95% CI should be provided so that intra- and intergroup variations can be easily ascertained. Although the SE of the median is less commonly encountered, it is defined as 1.253 times

the SE of the average (45), which is a common parameter. The 95% CI of the average is also a commonly used parameter; it is easily calculated by standard software programs (eg, Microsoft Excel, GraphPad Prism). Although the 95% CI of the median is less commonly calculated, it can be estimated by ranking the experimental values and then identifying the relevant upper and lower CI values based on their positions in the ranking. Tables for assigning the 95% CI values of the median based on the experimental group size have been published (47,48).

Data (M. G. Hollingshead, unpublished data) from the commonly used MDA-MB-361 subcutaneous tumor model illustrate a number of these issues. For example, an analysis of individual tumor weights for six tumors (Table 1) shows that at day 68 after implantation, the tumors showed substantial size heterogeneity, ranging from 63 to 3752 mg. The median tumor weight was 513 mg on day 68, and the average was 1151 mg. Examination of the individual tumor weights shows that the largest tumor (3752 mg) skewed the average tumor weight upward, whereas the median tumor weight tracked better with the individual tumor weights (Figure 4).

The importance of using a homogeneous tumor model is further demonstrated by the P values obtained from t tests comparing each day's tumor weights with the day 68 tumor weights (Table 1). With this highly heterogeneous model, the difference between the daily tumor weights never achieved a statistical significance level of .05. Thus, as seen here and in the power calculations described earlier, a tumor model with this degree of heterogeneity could not demonstrate a statistically significant difference, even when compared with the smallest possible tumor size (eg, the starting size of 14 mg). By contrast, data from MDA-MB-361 tumors grown in the mammary fat pad and staged to a starting median tumor weight of approximately 100 mg (Table 2) yielded a tumor size range on day 63 of 600–1504 mg across 13 mice. Although this range is large, the median and average tumor weights (1248 and 1105 mg, respectively) are consistent with each other. Moreover, in contrast to the heterogeneous tumor example, the median and average tumor growth curves (Figure 5) are similar and track well with the individual tumor data. Furthermore, the t test comparisons of tumor weights on day 63 with those on all the other measurement days indicate that the differences are statistically significant at multiple time points. This pattern is consistent with the power calculations described earlier, which had indicated that there was a 50% probability that group sizes of 12 would allow detection of a statistically significant difference between groups if their averages varied by 253 mg. In the example given here, a statistically significant difference was found between the day-53 and day-63 data points, with a P value of .03 and a difference in group averages of 263 mg.

Analysis of the individual and average relative tumor weights for these datasets indicates that relative tumor weights can be misleading because they do not provide insight as to the starting tumor mass. For example, the average 4.8 relative tumor weight on day 36 was 3.2 for subcutaneous MDA-MB-361 tumors (Table 1) suggests robust tumor growth. However, the initial tumor weight was 14 mg and the average and median tumor weights on day 36 were only 68 and 63 mg, respectively, which demonstrate that very little tumor growth occurred. Furthermore, the actual tumor weights indicate that the day 36 tumor size was just above the minimum size that can be reliably measured with calipers. By contrast, the relative tumor weight on day 36 was 3.8 for MDA-MB-361 cells implanted in the mammary fat pad (Table 2), and the median and average tumor weights were 325 and 347 mg, respectively. So, whereas comparing the day-36 relative tumor weights between Tables 1 and 2 would suggest that subcutaneously implanted MDA-MB-361 tumors grew more robustly than the same tumors in the mammary fat pad, comparisons of the median and average tumor weights indicate that tumors implanted in the mammary fat pad actually grew more quickly (Table 2).

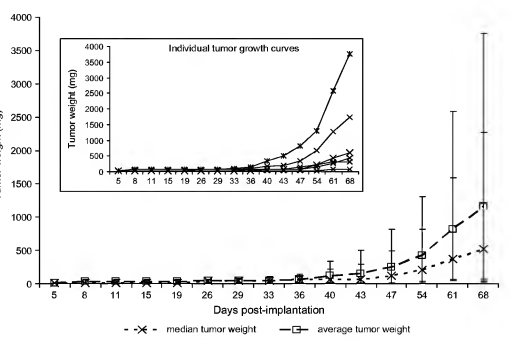

Figure 4. Tumor weight plots for MDA-MB-361 human breast tumors implanted subcutaneously in athymic nude mice. The data are from Table 1. The main graph presents the median and average tumor weights for a group of six mice (nu/nu Ncr; Animal Production Program, NCI-Frederick), each implanted with 1×10^7 cells in 0.1 mL. The inset presents the individual growth curve for each of the six mice. Individual tumor weights were calculated as weight in mg = (length × width^2)/2. The **error bars** indicate the 95% confidence intervals of the averages or the medians, as appropriate.

Appropriate Statistical Evaluation of Tumor Growth Data

Along with the specifics of the experimental protocol, efficacy data should use appropriate statistical evaluations to analyze the differences between the treated and control groups. The statistical tests used will vary with the experimental model, design, and endpoints collected (45). Many methods for selecting a relevant statistical test exist, but the best approach is to seek the assistance of a qualified statistician during the development of the experimental design. Alternatively, commercially available statistical analysis packages provide assistance to the investigator if a statistician is not available. In either case, selecting the statistical evaluation criteria for analyzing the data and defining the criteria for excluding outlying data points before conducting the experiment will remove the temptation to interpret the data in a manner that best supports the original hypothesis. An obvious example of this type of bias occurs when tumors that do not grow in the control group are excluded from the analysis because the operator expected them to grow, whereas tumor-free animals in the treated groups are included because the operator expected the treatment to work. Using a statistical test to identify which outliers should be excluded prevents this type of operator bias.

When selecting the statistical evaluation to be used for a dataset, the first criterion is whether the data have a normal or non-normal distribution. For normally distributed data, parametric tests such as the *t* test and analysis of variance are likely applicable. By contrast, non-normally distributed data generally require nonparametric tests such as the Wilcoxon, Mann–Whitney, or Kruskal–Wallis tests (46). The specific data to be analyzed will depend on the experimental design. For example, a tumor growing viscerally (eg, in the liver or kidney) may have only a single time point for data collection because the animal may have to be killed to collect and measure the tumor. By contrast, subcutaneously growing tumors have data from multiple time points. A common approach is to assess the statistical significance of differences in tumor size at each of the data collection times and to report the difference at the optimal time point. However, it is important to clarify that the statistical comparison presented is based on data for the optimal time point. A better approach is to present all of the data, along with the statistical differences found at each time point. If data for only a single time point are presented, then an explanation of how that time point was selected—whether because the differences were greatest then or for some other reason—should be provided.

When serial tumor growth data, such as for subcutaneously implanted tumors, are available, an alternative to presenting data for one or more individual time points is to compare the slopes of the tumor growth curves. Such slopes can be calculated readily with commercially available software programs. Although this data presentation method is not commonly used, it allows a statistical comparison of the growth rates for each of the experimental tumors, provided the tumor growth curves are reasonably monophasic. For example, using the data presented graphically in Figure 1, B, for MDA-MB-361 tumor xenografts, the average of the slopes for the vehicle control is 17.3 and that of the 45 mg/kg tamoxifen group is 0.78. The *P* value from the *t* test comparison of these slopes is less than .001. By contrast, the vehicle control group for MDA-MB-435 shown in Figure 1, A, had an average tumor growth slope of 20.2, whereas the group treated with 45 mg/kg tamoxifen had an average slope of 21.9, and the *P* value from the *t* test comparison of the slopes was .62. This approach is less valuable for tumors with multiphasic growth curves because the slope of the curve from the first to the last tumor measurement is not uniform.

When the experimental endpoint is modulation of molecular markers, then the investigator must determine which samples will be collected and analyzed. Valid samples may include tumor tissue, surrogate tissue (eg, spleen, bone marrow, skin), serum or plasma. Whatever the sample, a critical consideration in the experimental design is the timing of sample collection following exposure to the test agent. The method of collecting and storing the sample may also be critical to a reliable outcome because many markers are unstable and subject to change as a result of experimental conditions

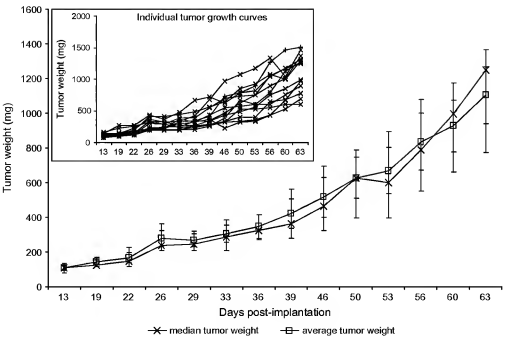

Figure 5. Tumor weight plots for MDA-MB-361 human breast tumors implanted in the mammary fat pads of athymic nude (nu/nu Ncr; Animal Production Program, NCI-Frederick) mice. The data are from Table 2. The main graph presents the median and average tumor weights for a group of 13 mice, each implanted with 1×10^7 cells in 0.1 mL. The inset presents the individual growth curves for each of the 13 mice. Individual tumor weights were calculated as weight in mg = (length × width^2)/2. The **error bars** are the 95% confidence interval of the average or the median, as appropriate.

(37,49). Performing assay optimization studies before conducting definitive therapeutic protocols will allow the collection method (eg, cryobiopsy, standard needle biopsy, and full or partial tumor resection) to be defined scientifically. In addition, the required sample size (amount of tissue needed to conduct the study) must be considered, along with the impact of the stability of the endpoint on the sample collection methodology. Part of the decision regarding sampling methods must consider the impact of pre- vs postmortem sampling, because some targets may be stable for several minutes postmortem, whereas others may degrade rapidly when respiration and perfusion cease.

If the endpoint being assessed is volatile or easily induced by stress or other manipulation of the host, then sample collection under general anesthesia (eg, using inhalation anesthetics such as isoflurane) may provide a higher quality, clinically relevant sample. Samples preserved in situ by cryobiopsy can be collected with commercially available clinical instruments such as the Cassi cryobiopsy needle. If in situ freezing is not required, then samples obtained by needle biopsy or resection can be placed into liquid fixative (eg, 10% neutral-buffered formalin and RNALater, Ambion, Austin, TX), flash frozen by transfer into a prefrozen cryovial, or stored in another appropriate manner. Whichever method is selected, it must be used consistently both within and across sample groups (37,49). The impact of operator fatigue should also be considered if large numbers of samples must be collected, so that the relative time of collection does not influence the results and subsequent conclusions.

Concluding Thoughts

The conclusions drawn from a series of studies are only as good as the data on which they are based. The impact of high-quality experimental design, methodology, and data interpretation cannot be overstated. To allow others to properly interpret the results of an in vivo efficacy study, it is important to provide a clear explanation of the experimental design, a reasonable overview of the data, and a scientifically justified interpretation of the data. Moreover, if appropriate consideration is given to the experimental design before animal studies are initiated, the number of animals used may ultimately be reduced, because multiple experiments may not be required. Along with a savings in animals there will likely be a concomitant savings in costs and time, contributing to an overall reduction in drug development costs. Conversely, poorly conducted experiments can produce misleading data that result in unnecessary additional studies that consume time, animals, and other resources. Although this in itself is wasteful and adds to the overall cost of drug development, perhaps the greater risk is the diversion of these important resources onto a fruitless course while other, better leads languish owing to a lack of resource availability.

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Notes

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Models of Anti-Cancer Therapy

Human Tumor Xenografts as Predictive Preclinical Models for Anticancer Drug Activity in Humans

Better Than Commonly Perceived—But They Can Be Improved

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ABSTRACT

It is not uncommon for new anti-cancer drugs or therapies to show highly effective, and sometimes even spectacular anti-cancer treatment results using transplantable tumors in mice. These models frequently involve human tumor xenografts grown subcutaneously in immune deficient hosts such as athymic (nude) or severe combined immune deficient (SCID) mice. Unfortunately, such preclinical results are often followed by failure of the drug/therapy in clinical trials, or, if the drug is successful, it usually has only modest efficacy results, by comparison. Not surprisingly, this has provoked considerable skepticism about the value of using such preclinical models for early stage in vivo preclinical drug testing. As a result, a shift has occurred towards developing and using spontaneous mouse tumors arising in transgenic and/or knockout mice engineered to recapitulate various genetic alterations thought to be causative of specific types of respective human cancers. Alternatively, the opinion has been expressed of the need to refine and improve the human tumor xenograft models, e.g., by use of orthotopic transplantation and therefore promotion of metastatic spread of the resultant 'primary' tumors.

Close inspection of retrospective and prospective studies in the literature, however, reveals that human tumor xenografts—even non metastatic ectopic/subcutaneous 'primary' tumor transplants—can be remarkably predictive of cytotoxic chemotherapeutic drugs that have activity in humans, when the drugs are tested in mice using pharmacokinetically clinically equivalent or 'rational' drug doses. What may be at variance with clinical activity, however, is the magnitude of the benefit observed in mice, both in terms of the degree of tumor responses and overall survival. It is argued that this disparity can be significantly minimized by use of orthotopic transplant/metastatic tumor models in which treatment is initiated after the primary tumor has been removed and the distant metastases are well established and macroscopic—i.e., the bar is raised and treatment is undertaken on advanced, high volume, metastatic disease. In such circumstances, survival should be used as an endpoint; changes in tumor burden using surrogate markers or micro-imaging techniques can be used as well to monitor effects of therapies on tumor response. Adoption of such procedures would more accurately recapitulate the phase I/II/III clinical trial situation in which treatment is initiated on patients with advanced, high-volume metastatic disease.

INTRODUCTION

One of the greatest challenges faced by developers of new drugs and treatment strategies for cancer is the obvious need to test them in preclinical in vivo models that have a good probability of being predictive of similar activity in humans. For more than half a century the laboratory mouse has been the primary species in which experimental cancer treatments have been tested. Until about 25 years ago syngeneic transplantable mouse tumors were used most commonly for such preclinical therapy studies, and still are, especially for immunotherapy experiments in which an intact immune system is required. The discovery that human tumor cell lines, and sometimes even primary biopsy human tumor specimens, can give rise to progressively growing, and potentially lethal cancers in immune deficient mice gradually resulted in a shift towards the use of human tumor xenografts for the study of virtually all other types of anti-cancer drugs and treatment strategies.[1] Essentially every clinically approved anti-cancer drug was tested using these models, and showed positive anti-cancer effects before being evaluated in early, and then late phase clinical trials. Nevertheless, these successes have been overshadowed by highly visible failures in which a particular new anti-cancer drug, or treatment strategy, demonstrated remarkable

anti-tumor effects using a transplantable tumor model in mice, only to be followed by failure in the clinical trial setting[2] ("failure" in this case being defined here as having little or no survival benefit, regardless of whether it was found to be safe, or not, in humans).

Perhaps the most spectacular and recent example of this was the study by Boehm, O'Reilly et al.[3] who reported stunning effects of endostatin on three different transplantable tumors subcutaneously grown in syngenic mice: the Lewis Lung carcinoma, the B16 melanoma and the T41 fibrosarcoma.[3] Cycles of daily endostatin treatment, an antiangiogenic protein drug, caused repeated and total regressions of established tumors. There was no evidence of relapse involving emergence of drug resistant variant/mutant subpopulations. Leaving aside the question of whether this result is reproducible (most other published studies of successful endostatin therapy show much more modest growth delays, but not overt tumor regressions), this result sparked enormous interest in both the scientific literature[4] and lay press.[5] It fueled unprecedented rapid initiation of phase I clinical trials in the United States, the results of which were recently reported.[6,7] The results of these trials showed the drug to be safe (which is the primary purpose of phase I trials) but there was certainly no evidence of the type of spectacular preclinical responses that had been observed in any of the treated patients.[6,7] The inevitable result has been the disappointment expressed not only about the drug itself, but about antiangiogenic therapy in general. In fairness, the results of other clinical trials involving antiangiogenic therapy such as the humanized monoclonal antibody to vascular endothelial cell growth factor (VEGF) known as bevacizumab (tradename: Avastin), which was tested in a randomized phase III trial as a third line therapy combined with Xeloda in advanced metastatic breast cancer, have also contributed significantly to this sense of current disappointment. But even in this case the disappointment stems, in part, from the many impressive results of prior preclinical studies utilizing a variety VEGF targeting of antiangiogenic drugs and approaches in a variety of mouse tumor models.

In 1999, Dr. Judah Folkman was quoted in a Newsweek magazine article as saying that a mouse study does not belong on the front page of the New York Times.[8] This makes considerable sense, and was a logical follow up to a quote he made in the May 3, 1998 Sunday New York Times article: "if you are a mouse and have cancer, we can take good care of you".[5] This statement would also seem to be logical, but as explained in this review, it is not necessarily so, and can be seriously challenged. Simply put, if you are a mouse with advanced, high-volume metastatic disease we probably cannot take good care of you.

The apparent lack of predictability of results often obtained using transplantable mouse or human tumors in normal or immune deficient mice has convinced many investigators to move away from such models and instead use spontaneously arising tumors, in particular genetically manipulated transgenic/knockout mice where the tumors which arise have murations thought to be causative of the respective human cancers.[9,10] Alternatively, other invesrigators have suggested that transplantable tumor models can be made much more predictive by orthotopic transplantation which frequently facilitates metastatic spread—especially of human tumor xenografts[11,12]—and thus testing the effects of a given therapy on either (or both) the primary tumor growing in a physiologically relevant site (as opposed to an ectopic site) and distant metastatic disease.

In this commentary, two major points are made:

1. growth and testing of human tumors in subcutaneous tissue sites that are ectopic for a given type of cancer have provided relevant and predictive information to the clinic, provided that clinically relevant, pharmacokinetic parameters (especially dosing) are employed; and,
2. orthotopic transplants are nevertheless potentially valuable when used to generate metastases—but that therapy should be initiated at a point when the metastases are well established and macroscopic in nature (i.e., high volume metastatic disease)—and not just on low-volume (occult) minimum residual disease, which is what almost all previous studies have utilized when testing therapies on metastatic disease.

Also highlighted is the need for continuous vigilance with respect to the nature and origin of the cell lines used for transplantable tumor studies.

RETROSPECTIVE STUDIES OF CHEMOTHERAPEUTIC DRUGS USING SUBCUTANEOUS/ECTOPIC HUMAN TUMOR XENOGRAFTS SHOWING A HIGH DEGREE OF CLINICAL RELEVANCE

Nomura, Inaba and colleagues of the Cancer Chemotherapy Centre, Japanese Foundation for Cancer Research, Tokyo, have published a series of important and insightful studies which show clearly the remarkable potential of ectopic human tumor xenografts for predicting the pattern of activity of conventional cytotoxic chemotherapeutic drugs in humans.[13-17] Prior to undertaking their studies many other published reports showed that the majority of chemotherapeutic drugs have significant anti-tumor effects on a particular type of human cancer, even though most of the drugs tested were known not to have such activity on the respective tumor type in the clinical situation. In other words, the results of preclinical xenograft models were not retrospectively predictive of clinical activity. However, Nomura, Inaba and colleagues reasoned this could be due to inappropriate drug dosing. It turns out that the maximum tolerated dose (MTD) of most chemotherapeutic drugs that be given to mice is higher (4–5 times) than in humans. In some cases, the MTD is lower in mice than in humans, and in some cases (e.g., adriamycin) it is the same. Thus, in many cases, if one uses the MTD of a given chemotherapeutic drug for mice, the blood levels of drug will be significantly higher than can be attained in humans, leading to false positive tumor responses in mice.

To study this hypothesis, Nomura, Inaba and colleagues tested a large number of independent cell lines (e.g., generally eight to twelve) for each type of cancer tested. They reasoned this was similar in nature to the number of patients in a typical phase I clinical trial, and as such, would minimize the risks associated with obtaining a false positive or false negative response when testing just a single or few cell lines. In other words, one looks for an overall pattern of response in mice to different drugs that may be similar to what is seem a population of cancer patients. Each tumor cell line was grown as subcutaneous xenograft in a number of athymic nude mice. These mice were subsequently treated with at least 5 or 6 different chemotherapeutic drugs, tested as monotherapies, where some of the drugs were known to be clinically active on the particular type of human cancer being tested, and some not. The drugs were administered to some groups of tumor-bearing mice using the MTD of the drug for mice, whereas in another group the pharmacokinetically clinically equivalent dose (CED) or "rational dose" for humans was used.

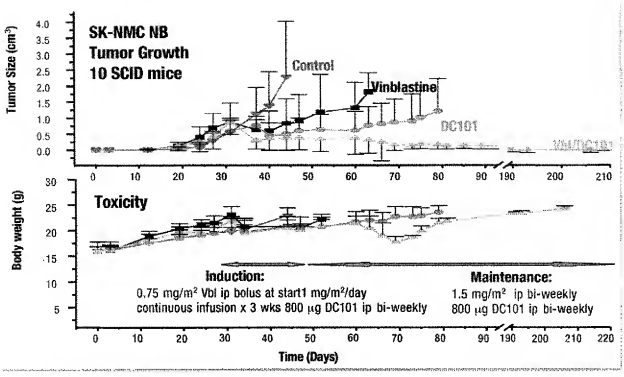

Figure 1. Results of an experiment in which large, established (0.75 cm^3) human neuroblastomas (NB) were treated with a metronomic low-dose vinblastine schedule, or DC101 (an anti-VEGFR-2 monoclonal antibody) or a combination of the two drugs. The dosing of the drugs is indicated in the lower figure. Note that the metronomic/ maintenance regimen was preceded by an induction regimen of the same drug to try and rapidly debulk the tumor mass before initiating the metronomic low-dose chemotherapy schedule. Progression of disease was seen in the single treatment groups, whereas slow but eventually complete tumor regression was noted in the combination group in which the therapy was continued for 7 months, which was possible by the lack of toxicity of this regimen. Taken from Klement, G. et al. "Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity" *J Clin Invest* 2000; 105:R15-R24.

Analysis of the data for a large number of tumor types including lung, glioma, breast and gastric cancers showed that the pattern of response obtained when the mouse MTD was used was not associated with clinical pattern of responsiveness—most or all drugs showed activity. In other words, there was a high rate of false positives. In striking contrast, when the clinically equivalent or rational dose was used, the pattern of response in mice was similar to the activity of the respective drugs in the respective human cancer.[13-18]

These results were obtained using over 60 different established human cancer cell lines, all of which were injected subcutaneously. In no case was orthotopic injection of a cell line undertaken.

PROSPECTIVE STUDIES USING SUBCUTANEOUS HUMAN CHILDHOOD TUMOR XENOGRAFTS

Houghton and colleagues at St. Judes Children's Hospital in Memphis have also undertaken an exhaustive series of pharmacokinetic investigations in which a variety of pediatric malignancies were tested as subcutaneous xenografts in nude mice with respect to response to a variety of chemotherapeutic drugs. In particular, the relationship between systemic exposure and tumor response was evaluated, with emphasis on topoisomerase inhibitors such as irinotecan or topotecan.[19-24] These studies showed that a panel of neuroblastoma xenografts was highly sensitive to irinotecan, especially when administered using protracted schedules with lower doses of drug. For example, irinotecan was administered intravenously (i.v.) daily 5 days per week for 2 consecutive weeks (defined as one cycle) and compared to more protracted low-dose schedules where cycles were repeated every 21 days for a total of three courses. In the latter the total amount of drug was 5–10 mg/kg and was given using a daily schedule for 5 days, which was repeated 2 out of every 3 weeks for 9 weeks. Complete responses were observed in most of four of five xenografts using the intensive one cycle 40 mg/kg MTD schedule but the tumors tended to regrow. In contrast, with one exception, all neuroblastomas tested showed complete responses (CRs) which did not regrow during therapy when the protracted low-dose schedules were used involving a total dose of 10 mg/kg or 5 mg/kg.[23] Estimation of the lowest effective dose using the protracted i.v. schedule indicated that neuroblastomas respond to daily doses as low as 1.25 mg/kg.[23] It is interesting to consider these results in the light of those obtained by other investigators using a variety of similar protracted low-dose "metronomic" chemotherapy regimens as a putative antiangiogenic therapy, where increased efficacy and reduced toxicity have been frequently noted using such schedules, compared to the MTD of the same drug.[25-31]

The preclinical studies of Houghton and colleagues were directly translated to the clinic where the same protracted schedule was used and found to be well tolerated in children with refractory solid tumors; in addition encouraging, if not remarkable, rates of clinical responses were observed as well—16 of 23 patients experienced

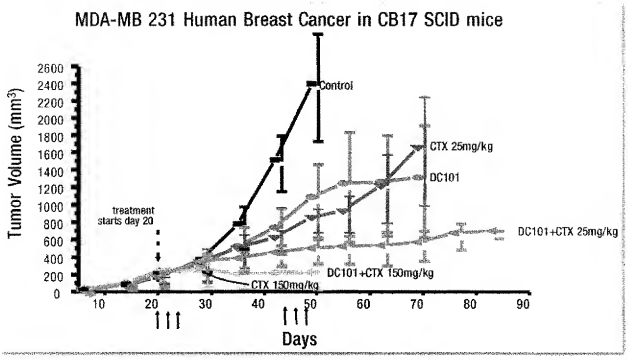

Figure 2. Results of an experiment published recently (Man et al. "Anti-tumor and anti-angiogenic effects in mice of low-dose (metronomic) cyclophosphamide administered continuously through the drinking water." Cancer Res., 62: 2731-2735, 2002) in which a human breast cancer cell line was injected "orthotopically" into the mammary fat pads of severe combined immunodeficient (SCID) mice, which allowed the tumor to metastasize to the lungs, liver and lymph nodes of the mice. Therapy was initiated when the "primary" intramammary fat pad tumor attained a size of 200 mm^3 and the disease had metastasized in a microscopic fashion only. Mice were then administered cyclophosphamide through their drinking water on a continuous non-stop basis at an estimated dose of 25 mg/kg per day, or treated with the DC101 anti-VEGFR-2 monoclonal antibody. In addition, another group of mice were given cyclophosphamide in the MTD fashion, i.e., at 150 mg/kg once every two days over a 6-day period (indicated by the vertical arrows). This MTD regimen was highly toxic to the SCID mice and resulted in death within one to two weeks. In contrast, mice given the same drug metronomically showed no signs of toxicity despite receiving up to 3 times the cumulative maximum tolerated dose given acutely.

stable disease and 5 showed partial responses.[24] These results show that preclinical xenograft models, even those involving ectopic/subcutaneous transplants, can provide useful predictors of the activity and responses of some pediatric cancers to topoisomerase I inhibitors such as irinotecan. A more detailed overview and discussion of the testing of new agents in childhood cancer models, both xenografts and transgenic oncomouse models was recently published by Houghton et al.[32]

IMPROVING HUMAN TUMOR XENOGRAFT MODELS FOR PREDICTING THE RELATIVE BENEFIT OF ANTI-CANCER DRUGS IN HUMANS—THE IMPORTANCE OF TREATING (ADVANCED) METASTATIC DISEASE

While the results summarized above are encouraging, and clearly show the potentially predictive value of human tumor xenografts, there is an aspect of the results in many of the preclinical studies that is nevertheless troubling: the excellent, if not remarkable, nature of the tumor responses in mice, as such responses are infrequently observed in cancer patients even though the drug being tested may be active against its respective human counterpart. For example, as discussed above, Houghton et al. observed complete responses of established solid neuroblastoma xenografts in a high proportion of cases using various irinotecan dosing schedules, especially protracted low-dose protocols.[25] However, such dramatic responses were not observed in the respective clinical trial of 23 patients, which included five children with neuroblastoma.[24] It is this aspect of experimental therapy studies in mice that can be frustrating as it often attracts considerable attention (e.g., the endostatin studies of Boehm, O'Reilly et al. discussed above) and expectation. This disparity has caused considerable skepticism about what to expect in the clinic on the basis of prior preclinical therapy studies. However, this skepticism may not always be justified when one takes into account, in retrospect, a crucial and fundamental difference between virtually all published experimental mouse therapy studies and corresponding clinical trials, and it is this: in most phase I, II and III clinical trials the patients being treated have advanced, high-volume metastatic disease whereas most mouse studies do not test the effects of therapy on advanced metastatic disease, but rather on a primary tumor transplant or spontaneously arising primary tumor, or microscopic, low-volume metastatic disease (Lee Ellis, personal communication). With respect to treatment of metastatic disease, typically, in such experiments, tumor cells are injected intravenously to generate lung or liver tumor colonies ("artificial metastases"), and therapy is initiated within one or a few days after injection of the cells—if not before tumor cell injection! This constitutes a form of adjuvant (or prophylactic) therapy, on microscopic, low-volume metastatic disease. Alternatively, growing primary tumors may be surgically removed, and treatment then initiated within a few days when the spontaneous metastases that have formed are microscopic in size. Thus, there is a much less demanding therapeutic situation for mice than for humans, when it

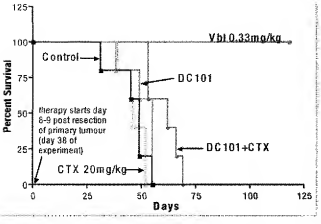

Figure 3. Effect of various therapy regimens on survival of SCID mice with advanced, metastatic cancer at the time of initiation of therapy. SCID mice were inoculated with MDA-MBA-435 human tumor cells. The inoculation was into the mammary fat pads which facilitated distant metastatic spread, provided the primary tumors are surgically excised. This was done approximately 4 weeks after tumor cell inoculation, after which therapy was initiated approximately 8-9 days later. The cyclophosphamide was given continuously through the drinking water at an estimated dose of 20 mg/kg per day, whereas vinblastine or taxol at the indicated dose were injected at low-dose twice a week.

comes to comparing most preclinical trials to the clinical trial counterparts. Perhaps much of the disparity in results between the two is related to this variable since it is well known that high-volume advanced metastatic disease is generally much more difficult to treat than low-volume adjuvant disease. Add to this the fact that many patients entered into clinical trials had been treated previously with other therapies and have relapsed with refractory disease. Heavily pretreated and resistant patients are often less responsive to a new therapy, and usually have advanced metastatic disease at the time of entry into a clinical trial.[33] How often have investigators in the past tested a new drug or therapy in mice where this dire clinical situation is recapitulated? The answer is rarely—if ever.

To illustrate the point about treating (advanced) metastatic disease, some recent results obtained in this laboratory are shown. Figure 1 shows the results of an experiment in which a metronomic low-dose vinblastine protocol, in combination with an antiangiogenic drug, called DC101 (an anti-VEGF receptor-2 blocking antibody) was used to treat large, established human neuroblastoma xenografts obtained after subcutaneous injection of SK-NM-C cells.[27] The results showed a remarkable anti-tumor effect could be obtained with the combination—sustained and complete tumor regressions. In effect, the mice were cured since the therapy was continuously maintained for 7 months,[27] and surprisingly, tumors did not resume growth when the treatment was finally terminated (unpublished observations). However, because the tumors were injected subcutaneously (i.e., ectopically) they did not metastasize, and therefore the much more demanding clinical situation of treating advanced metastatic neuroblastoma metastases was not duplicated in the mouse studies. The preclinical study was not intended to predict clinical activity—as implied by a headline proclaimed on the front page of a prominent national Canadian newspaper,[34] but to illustrate the principle of metronomic low-dose chemotherapy as a relatively non-toxic and effective way of giving chemotherapy, and combining it with a targeted antiangiogenic drug.[27,35-38]

Figure 2 shows the results of a similar experiment in which a human breast cancer (MDA-MB-231) was injected orthotopically in the mammary fat pads of female SCID mice, and then treated continuously with an oral low-dose regimen of cyclophosphamide administered continuously through the drinking water, combined with the same antiangiogenic drug, DC101.[39] A control using an MTD regimen of cyclophosphamide was also used. In terms of survival, the best treatment regimen was the combination of the metronomic oral low-dose cyclophosphamide/ DC101, and the survival benefit was obvious. However, in this model, while the orthotopic breast cancer can metastasize, the metastases remain largely microscopic because of the retention of the primary tumor and the timing of the initiation of treatment. Thus, treatment of low-volume, metastatic disease was undertaken.

More recent experiments have involved 'raising the therapeutic bar', so to speak. In Figure 3 a tumor cell line, called MDA-MB-435, supposedly a well known breast cancer cell line used extensively in breast cancer research, was injected into the mammary fat pads of SCID mice and allowed to grow for about one month. The resultant primary tumors were then surgically removed and initiation of treatment with oral low-dose cyclophosphamide and/or DC101 was delayed for about 10 days to allow establishment of extensive macroscopic metastases in the lungs and draining lymph nodes of the SCID mice, as well as diffuse metastatic spread in the liver (data not shown). Using survival as an endpoint, neither DC101 alone or oral low-dose cyclophosphamide alone had had impact on survival; the combination did have an effect, but the magnitude of the benefit was rather modest in comparison to the sort of results shown in Figures 1 and 2. Of considerable interest, however, was the finding that a metronomic low-dose vinblastine protocol—0.33 mg/kg given intraperitoneally three times a week—alone caused complete resolution of advanced metastatic disease and greatly prolonged survival of the mice. Eventually, the mice had to be sacrificed because tumors recurred at the site of surgical removal and grew progressively in spite of the success of the therapy on distant metastatic disease (unpublished observations).

It is of course difficult to compare the results of each experiment since different tumor cell lines and different treatment regimens were used. Indeed, the MDA-MB-435 'breast' tumor cell line has recently been implicated to be a melanoma, based on gene and protein expression profiling,[39,40] results which we have confirmed using the MDA-MB-435 line discussed in Figure 3. Nevertheless, the results of Figure 3 do suggest that treatment of advanced metastatic disease in mice will give results that may turn out to be much more reflective, i.e., predictive, of the clinical situation typically encountered when testing new drugs in phase I, II or III clinical trials. The vinblastine therapy results also point to the possibility that we cannot always assume that the response of a primary tumor will mirror the effects of the same therapy on distant metastases—this is obvious. What is not so obvious, and surprising, is that the response of metastases may be significantly better than the primary tumor in some cases. We would anticipate that this would be the exception rather than the rule; nevertheless this has ramifications for anti-cancer screening and drug testing, if correct.

CONCLUSIONS

In light of these results one might want to rethink Dr. Folkman's quote "if you are a mouse and have cancer, we can take good care of you".[5] One may argue this applies to mice with rapidly growing, transplanted, subcutaneous, encapsulated/non-metastatic tumors. In contrast, mice with high-volume, advanced, metastatic disease in sites such as the lungs, liver and brain may not be so easy to take care of, similar to their human counterparts. The vinblastine results do however provide some basis for optimism, and emphasize the need to begin testing models which involve advanced metastatic disease. This, incidentally, is one of the limitations of many of the current transgenic oncomouse models, as they usually do not spontaneously metastasize.[41,42] Moreover, monitoring the effects of therapies on metastatic disease in mice is becoming easier and less subjective with the growing use of small animal non-invasive micro-imaging research tools[43] and non-invasive biochemical techniques, e.g., measuring secreted tumor-specific protein markers that can be introduced into tumor cell lines.[29,44] It is also time to reexamine some of the current dogmas regarding mouse models of cancer. First, human tumor xenografts can be surprisingly predictive of clinical activity, and in some cases this includes subcutaneous/ectopic transplants. The wisdom of the rush towards exclusive use of much more expensive transgenic oncomouse models for drug therapy testing can be questioned, especially when such tumors fail to express the most critical element of malignant disease—ability to metastasize, and the fact that less expensive transplantable tumor models are available which work—if used appropriately.

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KChAP as a chaperone for specific K^+ channels

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Kuryshev, Yuri A., Tatyana I. Gudz, Arthur M. Brown, and Barbara A. Wible. KChAP as a chaperone for specific K^+ channels. *Am J Physiol Cell Physiol* 278: C931–C941, 2000.—The concept of chaperones for K^+ channels is new. Recently, we discovered a novel molecular chaperone, KChAP, which increased total Kv2.1 protein and functional channels in *Xenopus* oocytes through a transient interaction with the Kv2.1 amino terminus. Here we report that KChAP is a chaperone for Kv1.3 and Kv4.3. KChAP increased the amplitude of Kv1.3 and Kv4.3 currents without affecting kinetics or voltage dependence, but had no such effect on Kv1.1, 1.2, 1.4, 1.5, 1.6, and 3.1 or Kir2.2, HERG, or KvLQT1. Although KChAP belongs to a family of proteins that interact with transcription factors, upregulation of channel currents was not blocked by the transcription inhibitor actinomycin D. A 98-amino acid fragment of KChAP binds to the channel and is indistinguishable from KChAP in its enhancement of Kv4.3 current and protein levels. Using a KChAP antibody, we have coimmunoprecipitated KChAP with Kv2.1 and Kv4.3 from heart. We propose that KChAP is a chaperone for specific Kv channels and may have this function in cardiomyocytes where Kv4.3 produces the transient outward current, I_{to}.

Kv2.1; Kv4.3; Kv1.3; protein inhibitor of activated STAT3; rat heart

VOLTAGE-DEPENDENT POTASSIUM CHANNELS (Kv) contribute to the electrical properties of excitable cells such as cardiomyocytes and neurons by affecting action potential shape and duration. Kv channels are also important in many nonexcitable cell types where they may be involved in diverse processes such as secretion, cell volume regulation, and activation by mitogens. Four gene families encompassing a total of at least 16 different genes encode the majority of the pore-forming Kvα-subunits (Kv1–4) (15). Kvα-subunits assemble as functional tetramers with multiple members of each subfamily exhibiting tissue-specific expression. Given the extensive diversity in Kv channels, matching native K^+ currents with the correct Kv gene product has been problematic. In some cases though, there is a good correlation between native K^+ current and Kv channel. For example, Kv4.3 is thought to underlie the transient outward current (I_{to}) in adult heart (8, 9, 16), whereas Kv1.3 has been shown to be responsible for the *n*-type current characteristic of T lymphocytes (5).

The level of expression and/or functional properties of certain Kv channels can be modulated by accessory cytoplasmic proteins. To date, two distinct types of modulatory proteins have been described: Kvβ-subunits and KChAP. Multiple Kvβ-subunits that modulate the kinetics and/or expression of channels in the Kv1 subfamily have been described (1, 2, 10, 11, 20, 22, 24, 26, 27, 29, 33). These subunits bind tightly to the amino termini of Kvα-subunits and, although they have been described as chaperone-like (26), they are found at the cell surface in native channel complexes. Kv1 and Kv2 α-subunits also bind a cytoplasmic Kv channel-associated protein, KChAP, which we cloned and characterized recently (30). This association occurs at the intracellular amino terminus of the Kvα-subunit, but, unlike Kvβ-subunits, KChAP does not remain attached to the channel at the cell surface. Coexpression of KChAP with Kv2.1 in *Xenopus* oocytes produced a significant increase in both total Kv2.1 channel protein and the number of functional Kv2.1 channels at the cell surface. Our observations led to the hypothesis that KChAP acts as a chaperone to enhance expression of Kv2.1 protein.

KChAP belongs to a newly described multigene family consisting of PIAS3 (6), GBP (28) or PIAS1 (19), and Miz1 (31) or ARIP3 (21). PIAS3, GBP/PIAS1, and Miz1/ARIP3 were all cloned in the yeast two-hybrid system as transcription factor interacting proteins. PIAS3, which is most homologous to KChAP, is thought to bind to activated STAT3, resulting in the inactivation of this transcription factor (6). Thus KChAP is likely to have multiple cellular binding partners in addition to Kv channels.

The present experiments were designed to *1*) determine the number of Kv channels with which KChAP interacts, *2*) explore the possibility of transcriptional activation by KChAP, *3*) identify the KChAP domain mediating chaperone effects, and *4*) examine KChAP/Kv channel interactions in native cells. The results show that KChAP enhances the functional expression, without altering gating or voltage dependence, of a subset of Kv channels, Kv1.3, Kv2.1, and Kv4.3, without increasing Kv1.1, 1.2, 1.4, 1.5, 1.6, 3.1, or Kir2.2, HERG, or KvLQT1 currents. The effects are independent of transcriptional activation, and a 98-residue fragment is able to reproduce both the binding and current enhancement effects of full-length KChAP. KChAP may chaperone Kv channels in cardiomyocytes, since complexes of KChAP and Kv2.1 or Kv4.3 were coimmunoprecipitated from rat heart.

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METHODS

In Vitro Transcription of cRNAs and Oocyte Injection

Kv4.3 was obtained by RT-PCR from rat brain RNA and subcloned into pSP64 for cRNA synthesis. Mouse Kv 1.3 cDNA in pSP64 was purchased from the American Type Culture Collection. KChAP with an amino-terminal extension of 45 amino acids (MVMSFRVSELQVLGFAGRNKSGRKHEL-LAKALHLLKSSCAPSVQ) was amplified by PCR from our original rat brain cDNA clone and subcloned into pSP64. We had originally assigned KChAP methionine-46 as the initiating residue based on the presence of an in-frame upstream stop codon (30). After a DNA sequencing error upstream of this position was discovered, which removes this stop codon, it now appears likely that the start site is 45 residues upstream. The original KChAP as well as KChAP with the amino-terminal extension exhibit no differences in their binding in yeast two-hybrid assays or in their effect on Kv channels in heterologous expression assays. cRNAs were prepared and injected as previously described (23, 30).

Constructs and Yeast Two-Hybrid Assay

Full-length KChAP (residues 1–619) and the following KChAP fragments were tagged with enhanced green fluorescent protein (EGFP) at their amino termini by subcloning into the *Eco*R I and *Sal* I sites of EGFP-C2 (Clontech): KChAP-N (residues 46–354), KChAP-M (residues 355–452), and KChAP-C (residues 453–619). The same fragments were also subcloned into the GAL4 yeast two-hybrid vectors, pGBT9 and pGAD424. Fragments encoding Kv 1.2-N (residues 1–164) and Kv 1.3-N (residues 1–186) were also subcloned into pGBT9. Protein-protein interactions were tested in the yeast two-hybrid system by cotransformation of host strain *HF7C* with pairs of pGBT9 and pGAD424 fusion constructs as previously described (23). Cotransformants were selected and spotted on media with and without histidine to follow the activation of the *HIS3* reporter gene.

Cell Lines and Injection

Mouse L cells were grown in minimum essential medium with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies). L cells stably transfected with either Kv 1.1, Kv 1.5, Kv4.3, HERG, or KvLQT1 + minK cDNAs were isolated, using methods as previously described in Ref. 7, and maintained in media containing 0.5 mg/ml G418 (Life Technologies). L cells were injected with an Eppendorf 5242 microinjector and 5171 micromanipulator (Madison, WI) as described previously (14). The bottoms of 35-mm plastic dishes were labeled with circles (1 mm diameter), and cells were plated at a density of ~20 cells/circle.

Transient Transfection and Immunocytochemistry

Transient transfection. L cells stably expressing Kv4.3 (L-Kv4.3) were plated 1 day before transfection on poly-L-lysine-coated coverslips. For transient transfection with EGFP-C2, EGFP tagged full-length KChAP or KChAP fragments, cDNAs were mixed in a 1:5 ratio with Lipofectin (Life Technologies) and incubated with the cells for 4 h at 37°C. In some experiments, polyethylenimine (Fluka) was used as the lipid carrier (4).

Immunofluorescence microscopy. About 48 h post-transfection, cells were fixed in 4% paraformaldehyde in PBS for 15 min at room temperature and permeabilized for 5 min with 0.1% Triton X-100 in PBS. After blocking for 1 h in 3% nonfat milk-PBS, the cells were incubated overnight at 4°C with Kv4.3 polyclonal antibody (1:200 dilution; Alomone Labs) in 3% nonfat milk PBS. The secondary antibody, tetramethylrhodamine B isothiocyanate (TRITC)-conjugated anti-rabbit IgG, (1:100; Jackson Labs), was added for 1 h at room temperature. Coverslips were mounted with Vectashield (Vector Labs) and examined with an Olympus BH-2 microscope. Images were obtained with a Spot 2.1 digital camera (Diagnostic Instruments).

Electrophysiology

Measurements of *Xenopus* oocyte whole cell currents were performed using the standard two-microelectrode voltage-clamp technique as described previously (30). In mouse L cells, K^+ currents were measured using the whole cell configuration of the gigaseal recording technique (13). The pipette solution contained (in mM) 140 potassium aspartate, 5 $MgCl_2$, 10 HEPES, 10 EGTA, 10 glucose, and 2 Na_2ATP at pH 7.2. The bath solution contained (in mM) 140 NaCl, 5.4 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, and 10 glucose at pH 7.4.

Data acquisition and analyses were performed with pCLAMP 5.5.1 software (Axon Instruments, CA). In oocytes, linear leakage and capacity transient currents were subtracted using a P/4 prepulse protocol. Records were low-pass filtered at 2 or 5 kHz and digitized at 10 kHz. Experiments were conducted at room temperature (20–22°C). Data are reported as means ± SE. Comparisons between two groups of cells were performed by *t*-test. Comparisons between multiple groups of cells were performed by one-way ANOVA test and Student-Newman-Keuls post hoc test. Means are considered to be significantly different when $P < 0.05$.

Tissue and Oocyte Lysate Preparation

Freshly dissected adult rat hearts (Sprague-Dawley) were minced and placed into ice-cold lysis buffer (1:7 wt/vol) containing (in mM) 150 NaCl, 50 Tris, 1 EDTA, 0.2% BSA, 1% Triton X-100, pH 7.5, supplemented with a protease and phosphatase inhibitor cocktail (Complete; Boehringer-Mannheim; plus 50 mM sodium fluoride and 0.2 mM sodium vanadate). Samples were homogenized with a Polytron at setting 6 for 5 s. After a 1-h incubation on ice, the lysates were centrifuged at 900 *g* for 10 min to remove insoluble material. *Xenopus* oocytes were homogenized with 20 strokes in a glass homogenizer in lysis buffer (20 μl/oocyte) and incubated on ice for 1 h, and insoluble debris was removed by centrifugation at 3,000 *g*. Protein concentrations were determined by the bichinchoninic acid method (Pierce).

KChAP Antibody Production, Western Blotting, and Immunoprecipitation

Antibody production. A bacterial fusion protein consisting of maltose-binding protein (MBP; New England Biolabs) and the carboxy terminus of KChAP (residues 453–619) was purified on amylose resin and sent to Research Genetics for polyclonal antibody production. IgG was purified on a protein G Sepharose column (Pharmacia) and passed over an MBP affinity column, and anti-KChAP reactivity was immunopurified on a KChAP:MBP affinity column.

Polyclonal antisera were also raised to a peptide (residues 534–549, SPSVITSLDEQDTLGH) in the carboxy terminus of KChAP (SynPep). The antibody, referred to as KChAP-Ctpep, was affinity purified on an AminoLink column (Pierce) to which free peptide was coupled.

Western blotting. Lysates and solubilized immunoprecipitated proteins were separated on 10% SDS polyacrylamide gels and blotted to polyvinylidene difluoride membranes. After blocking with 5% nonfat dry milk in PBS plus 0.1% Tween 20 (PBST), blots were incubated with primary antibodies for 1 h at room temperature. The blots were washed three times in PBST at least 30 min, incubated in horseradish

peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech; 1:3,000 dilution in 5% nonfat dry milk /PBST) for 1 h at room temperature and washed three times in PBST. The blots were developed with the ECL+Plus detection system (Amersham Pharmacia Biotech).

Immunoprecipitation. Immunoprecipitation reactions were performed at 4°C using rat heart lysates. For each experiment, the lysate (2–4 mg/ml) was equally divided into two tubes. Lysates were precleared by incubation with anti-rabbit IgG-conjugated magnetic beads (Dynabeads; Dynal), after which affinity-purified anti-KChAP was added to one tube (1:100 dilution). Both tubes were incubated overnight with gentle mixing at 4°C. Antigen-antibody complexes were captured on Dynabeads by gentle mixing for 1 h at 4°C. The Dynabeads were washed three times with lysis buffer, and immunoprecipitates were eluted by boiling in reducing SDS sample buffer.

RESULTS

KChAP Increases the Functional Expression of Kv1.3 and Kv4.3 in Xenopus Oocytes

We showed previously that KChAP produced significant increases in Kv2.1, but not Kv1.5, currents when coexpressed in *Xenopus* oocytes (30). Here we examined the effect of KChAP on other Kv channels when both cRNAs were expressed in *Xenopus* oocytes. Kv1.3 current amplitude was increased about twofold when the channel was coexpressed with KChAP (Fig. 1*A*). This increase occurred in the absence of changes in channel gating or voltage dependence of activation (Fig. 1*B*). Activation (from −80 to +70 mV) and deactivation (from +70 to −80 mV) time constants of currents in

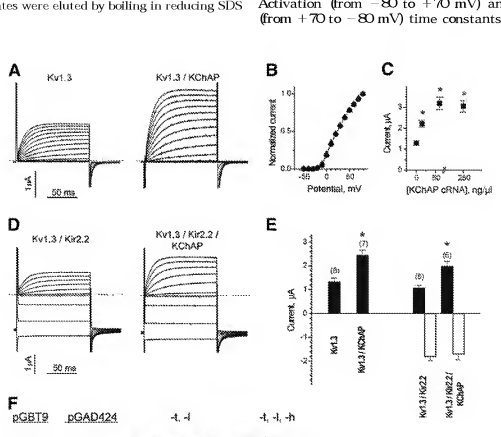

Fig. 1. KChAP increases the functional expression of Kv1.3 in *Xenopus* oocytes. *A*: whole cell currents measured 24 h after injection in oocytes injected with Kv1.3 cRNA (0.5 ng/μl) alone (*left*) or coinjected with KChAP cRNA (12.5 ng/μl; *right*). Holding potential was −80 mV, and 100-ms pulses were from −70 to +80 mV in 10-mV steps; 50 mM K^+ in bath solution. *B*: normalized and averaged peak currents plotted as a function of test potential in oocytes (same injection series as in *A*) injected with Kv1.3 cRNA alone (0.5 ng/μl; ●) or + KChAP cRNA (12.5 ng/μl; ▲). *C*: dose dependence of KChAP cRNA on Kv1.3 expression. Current amplitudes were measured at pulses to +70 mV. Oocytes were injected with Kv1.3 cRNA (0.5 ng/μl) alone or coinjected with increasing amounts of KChAP cRNA (1.5, 6.2, and 23.0 ng/μl; $n = 10$ for each concentration). *D*: whole cell currents measured 24 h after injection in oocytes coinjected with Kv1.3 cRNA (0.5 ng/μl) and Kir2.2 cRNA (10 ng/μl; *left*) or coinjected with KChAP cRNA (12.5 ng/μl; *right*). Holding potential was −80 mV, and 100-ms pulses were from −90 to +50 mV in 20-mV steps; 50 mM K^+ in bath solution (same batch of oocytes as in *A*). *E*: bar plot of averaged currents shown in *A* and *D*. Currents through Kv1.3 channels (solid bars) were measured at +70 mV, and currents through Kir2.2 channels (open bars) were measured at −70 mV. Numbers of oocytes are indicated in parentheses above bars. *Significant difference from currents in oocytes without KChAP (*C* and *E*). *F*: yeast two-hybrid assay of interaction of Kv1.3N with KChAP. Yeast host strain *HF7c* was cotransformed with GAL4-binding domain (pGBT9) and activation domain (pGAD424) fusion plasmids as indicated, and initially plated on media lacking tryptophan and leucine (−t, −l). Three individual colonies from each cotransformation were respotted on media with (−t, −l) or without histidine (−t, −l, −h) to follow activation of the *HIS3* reporter gene as shown on *right*. Growth on media without histidine is indicative of an interaction.

oocytes injected with Kv1.3 cRNA alone were 4.0 ± 0.1 and 13.6 ± 0.2 ms (n = 16), respectively, whereas, in oocytes coinjected with Kv1.3 and KChAP cRNAs, the time constants were 4.1 ± 0.1 and 13.3 ± 0.3 ms (n = 15), respectively. The effect of KChAP on Kv1.3 current amplitude was dose dependent (Fig. 1*C*), with increasing concentrations of KChAP cRNA producing increasing currents until saturation was reached. We reported previously that KChAP had no effect on Kir2.2 currents (an inwardly rectifying K^+ channel) (30). As an additional control of KChAP specificity, we coinjected oocytes with a mixture of Kv1.3 and Kir2.2 cRNAs with or without KChAP. In the presence of KChAP, outward current from Kv1.3 channels was significantly increased, whereas, in the same oocytes, the amplitude of the inward current from Kir2.2 channels was unchanged (Fig. 1, *D* and *E*).

We demonstrated previously that KChAP was able to bind to the amino terminus of both Kv2.1 and Kv1.5 channels (30). Because we saw a significant increase in Kv1.3 currents in the presence of KChAP, we tested the amino terminus of Kv1.3 for its ability to bind to KChAP. A yeast two-hybrid interaction assay indicated that Kv1.3-N was able to interact with KChAP. As shown in Fig. 1*F*, growth on minus histidine selection media as a result of the activation of the *HIS3* reporter gene was indicative of interaction between the two proteins.

Kv1.3 is unique, among the Kv1 subfamily members that we have studied, in its sensitivity to KChAP. A thorough examination by heterologous expression in *Xenopus* oocytes revealed no enhancement of Kv1.2, Kv1.4, Kv1.5, or Kv1.6 current amplitudes by KChAP (Fig. 2), even though the amino termini of Kv1.2, Kv1.4, and Kv1.5 interacted with KChAP in yeast two-hybrid assays (30). We expanded our analysis to additional Kv subfamilies and found that Kv3.1 currents were unaffected by KChAP but that the amplitude of Kv4.3 currents increased significantly (Fig. 2). Due to nonspecific transactivation of the reporter genes by Kv3.1-N and Kv4.3-N in yeast two-hybrid assays, we were not able to use this method to determine whether KChAP interacted directly with either channel. As a further check of its specificity, KChAP did not increase Kir2.2, HERG, or KvLQT1 currents when tested in oocytes (Fig. 2).

An examination of the effects of KChAP on Kv4.3 currents in *Xenopus* oocytes is presented in Fig. 3. In the presence of KChAP, Kv4.3 currents were increased about twofold (Fig. 3*A*), while the kinetics of activation and inactivation were unchanged (Fig. 3, *B* and *C*). At depolarizing test potentials from −80 to +70 mV, activation constants were 1.6 ± 0.3 and 1.6 ± 0.2 ms for currents recorded from oocytes injected with Kv4.3 cRNA alone (n = 24) and Kv4.3 plus KChAP cRNAs (n = 24), respectively. Inactivation constants were 40.1 ± 0.3 ms (Kv4.3 alone; n = 24) and 40.8 ± 0.4 ms (Kv4.3 + KChAP; n = 24). The values of half-maximal inactivation ($V_{0.5}$) were −45.6 ± 0.6 and −46.8 ± 0.2 mV, and the slope factors (k) were −5.8 ± 0.1 and −6.0 ± 0.1 mV for currents from oocytes injected with Kv4.3 cRNA alone (n = 5) and Kv4.3 plus KChAP cRNAs (n = 5), respectively. As with Kv1.3, KChAP

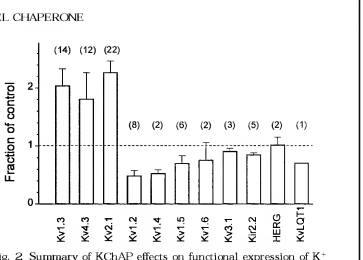

Fig. 2 Summary of KChAP effects on functional expression of K^+ channels in *Xenopus* oocytes. Bar plot showing average results from independent injection series. Numbers above bars indicate number of injection series (different batches of oocytes) for each K^+ channel. Oocytes were injected either with K^+ channel cRNA alone (control) or + KChAP cRNA. In each series, currents from 6–12 oocytes were measured, and ratio of means (current of coinjected/current of control) was calculated. Peak (Kv4.3 and Kv1.4) or steady-state (other Kv channels) currents were measured at a test potential of +70 mV (5 or 50 mM K^+ in bath). Kir2.2 steady-state and HERG tail currents were recorded at test potentials to −100 mV with a prepulse to +20 mV (50 mM K^+ in bath). KvLQT1 currents were measured at the end of a 2-s test pulse to +40 mV. Measurements were made on *postinjection day 1* for Kv1.3 and Kv1.6; *day 2* for Kv1.2, Kv3.1, Kv4.3, Kir2.2, and KvLQT1; and *days 5*or *6* for Kv1.4, Kv1.5, Kv2.1, and HERG. Coexpression of KChAP with Kv1.3 produced significant increased current amplitude in 10 of 14 batches of oocytes (in every batch, currents were measured in 6–10 control and coinjected oocytes). Significant increases were obtained in 7 of 12 batches of oocytes (for Kv4.3) and 18 of 22 batches (for Kv2.1). Average increases in all tested batches were 2.04 ± 0.23, 2.27 ± 0.20, and 1.87 ± 0.45 times for Kv1.3, Kv2.1, and Kv4.3, respectively. Magnitude of the KChAP effect was dependent on the particular batch of oocytes, but all 3 channels behaved similarly when examined in the same batch of oocytes. Current increases were never observed when KChAP was tested with Kv1.2, Kv1.4, Kv1.5, Kv1.6, Kv3.1, Kir2.2, HERG, or KvLQT1. In fact, coinjection with KChAP resulted in significant current suppression for Kv1.2 (in 7 of 8 oocyte batches) and Kv1.4 (2 of 2).

expression enhancement of Kv4.3 currents was dose dependent and saturable with increasing amounts of KChAP cRNA (Fig. 3*D*). Coexpression with KChAP did not alter the time course of Kv4.3 expression in oocytes that peaked at *day 2* and decreased thereafter (Fig. 3*E*), suggesting that, if there were more Kv4.3 channels at the cell surface as for Kv2.1 (30), their stability at the surface was not altered.

KChAP Increases Kv2.1, Kv4.3, and Kv1.3 Currents in Transfected Mammalian Cells

Thus far, all of our functional assays of KChAP have been in *Xenopus* oocytes. We wanted to determine whether the oocyte observations could be replicated in mammalian cells. In the first set of experiments, KChAP and Kv2.1 cRNAs were microinjected into mouse L cells. Noninjected cells or cells injected with 100 mM KCl had very small outward currents, whereas cells injected with Kv2.1 cRNA exhibited voltage-dependent outwardly rectifying current (Fig. 4*A*). The current density in cells coinjected with a mixture of Kv2.1 and KChAP cRNAs was significantly higher then in cells injected with Kv2.1 cRNA alone (Fig. 4, *A* and *B*).

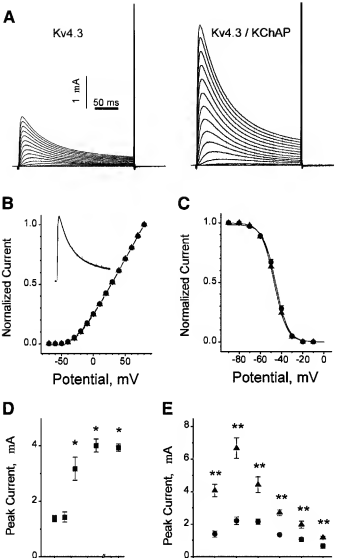

Fig. 3 KChAP increases functional expression of Kv4.3 in *Xenopus* oocytes without affecting activation or inactivation properties. *A*: whole cell currents measured *postinjection day 2* in oocytes injected with Kv4.3 cRNA (10 ng/nl) alone (*left*) or coinjected with KChAP cRNA (300 ng/nl; *right*). Holding potential was −90 mV, and 200-ms pulses were from −70 to +80 mV in 10-mV steps; 5 mM K^+ in bath solution. *B*: normalized and averaged peak currents plotted as a function of test potential in oocytes (same injection series as in *A*) injected with Kv4.3 cRNA alone (10 ng/nl; ●) or + KChAP cRNA (300 ng/nl; ▲). *Inset*: superimposition of averaged and normalized currents from oocytes injected with Kv4.3 alone and Kv4.3 + KChAP at +70 mV test potential. *C*: steady-state inactivation curves of currents from oocytes injected with Kv4.3 cRNA alone (n = 5; ●) or + KChAP cRNA (n = 5; ▲). *D*: dose dependence of KChAP cRNA on Kv4.3 expression. Peak current amplitudes were measured at pulses to +70 mV. Oocytes were injected with Kv4.3 cRNA (10 ng/nl) alone or with increasing amounts of KChAP cRNA (3.1, 6.2, 12.5 and 50.0 ng/nl; n = 12 for each concentration; same injection series as in *A* and *B*). *Significant difference from value for Kv4.3 alone. *E*: time dependence of KChAP effect on Kv4.3 channel expression in 1 injection series. Peak currents were measured at a pulse to +70 mV from oocytes injected with Kv4.3 cRNA alone (10 ng/nl; ●, n = 10 for each point) or + KChAP cRNA (23.0 ng/nl; ▲, n = 10 for each point). **Significant difference from Kv4.3 alone on same day after injection.

To examine the effect of KChAP on Kv4.3 in mouse L cells, we transiently transfected a cell line stably expressing Kv4.3 (L.Kv4.3) with a plasmid encoding a chimeric protein consisting of KChAP fused to the carboxy terminus of EGFP (EGFP-KChAP). As a control, the plasmid EGFP-C2 was transfected. Cells exhibiting green fluorescence were chosen for electrophysiological recording. L.Kv4.3 cells exhibited a relatively small transient outward current (Fig. 4*C*). Kv4.3 currents were not altered in cells coexpressing EGFP (Fig. 4*C*). However, cells transfected with the chimeric EGFP-KChAP construct exhibited dramatically increased (8- to 10-fold) currents with no apparent changes in voltage dependence or channel gating (Fig. 4, *C* and *D*). The Kv4.3 currents in cells coexpressing EGFP or EGFP-KChAP had similar kinetics of activation and inactivation. With pulses from −90 to +40 mV, activation time constants were 4.09 ± 0.21 ms (EGFP; n = 16) and 3.92 ± 0.36 ms (EGFP-KChAP; n = 16). Inactivation time constants were 79.3 ± 5.4 ms (EGFP; n = 16) and 72.8 ± 4.0 ms (EGFP-KChAP; n = 16). Parameters of steady-state inactivation were also similar: $V_{0.5}$ = −49.4 ± 0.8 mV, k = −6.7 ± 0.2 mV (n = 5); and $V_{0.5}$ = −51.5 ± 3.0 mV, k = −7.2 ± 0.4 mV (n = 5) for EGFP and EGFP-KChAP, respectively. Recovery from inactivation did not differ in cells coexpressing EGFP (recovery constant at −100 mV; 21.7 ± 1.8 ms; n = 6) or EGFP-KChAP (19.4 ± 2.5 ms; n = 4).

Because the mammalian cell environment supported the action of KChAP on Kv2.1 and Kv4.3 currents as well or better than *Xenopus* oocytes, we tested the effect of KChAP on Kv1.5 in L cells, a channel whose amplitude is not modulated by KChAP in oocytes. An L cell line stably expressing Kv1.5 (L.Kv1.5) was transiently transfected with either EGFP-C2 or EGFP-KChAP. As shown in Fig. 4, *E* and *F*, there was no significant alteration in the amplitude of Kv1.5 currents in any of the transiently transfected cells. Thus KChAP did not modulate Kv1.5 in either oocytes or L cells.

A summary of the effects of KChAP on K^+ channels in L cells is presented in Fig. 4*G*. In addition to Kv2.1, Kv4.3, and Kv1.5, we also examined the effect of KChAP on four other stable L cell lines: L.Kv1.1, L.Kv1.3, L.HERG, and L.KvLQT1 + minK. As with Kv1.5, Kv1.1 currents were unaffected by the coexpression of KChAP. Neither HERG nor KvLQT1 + minK currents were altered by KChAP in L cells. However, Kv1.3 currents were increased by KChAP in mammalian cells as well as oocytes.

Kv α-Subunit Binding Region Is Localized to a Stretch of 98 Residues in KChAP

To define the Kv channel binding region of KChAP, KChAP was divided into three fragments and each was tested in the yeast two-hybrid system for interaction with the amino terminus of Kv1.2 (Kv1.2-N). The KChAP fragments are diagrammed in Fig. 5*A*, and the results of the yeast two-hybrid assay are presented in Fig. 5*B*. Full-length KChAP as well as KChAP-M, consisting of a stretch of 98 residues in the middle of the protein (amino acids 355–452), interacted with

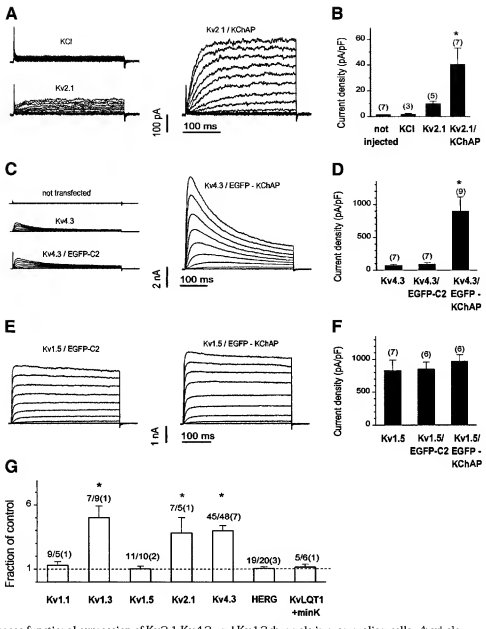

Fig. 4. KChAP increases functional expression of Kv2.1, Kv4.3, and Kv1.3 channels in mammalian cells. *A*: whole cell currents in L cells injected with either 100 mM KCl, Kv2.1 cRNA (12.5 ng/μl), or Kv2.1 + KChAP cRNAs (12.5 and 25.0 ng/μl, respectively). Holding potential was −80 mV, and 300-ms pulses were from −70 to +70 mV with 10-mV steps. Recordings were made 1 day after injection. *B*: bar plot showing effect of KChAP on Kv2.1 current density at +70 mV. *Significant difference from cells injected with Kv2.1 cRNA alone. Numbers of cells are indicated above bars. *C*: whole cell currents in untransfected L cells, in L cells stably transfected with Kv4.3 channels (L Kv4.3 cells), in L Kv4.3 cells transiently transfected with enhanced green fluorescent protein (EGFP)-C2, and in L Kv4.3 cells transiently transfected with EGFP-KChAP. Recordings were made 2 days after transfection. Holding potential was −80 mV; 400-ms pulses were from −70 to +40 mV in 10-mV steps. *D*: effect of EGFP-KChAP on Kv4.3 current density at +40 mV. *Significant difference from current density in L Kv4.3 cells and L Kv4.3 cells transiently transfected with EGFP-C2. *E*: whole cell currents in L Kv1.5 cells transiently transfected with EGFP-C2 or EGFP-KChAP. Voltage protocol was same as for Kv4.3. *F*: current density in L Kv1.5 cells and L Kv1.5 cells expressing EGFP or EGFP-KChAP at +40 mV. *G*: summary of KChAP effects on functional expression of K^+ channels in L cells. In case of Kv2.1 channels, L cells were coinjected with both cRNAs. For Kv1.1, Kv1.5, Kv4.3, HERG, and KvLQT1 + minK, stably transfected cell lines were used and cells were transiently transfected either with EGFP-C2 (control) or EGFP-KChAP plasmids. Numbers above bars indicate number of analyzed cells: KChAP-transfected cells/control cells. Number in parentheses refers to number of independent transfection experiments. Whole cell control currents were averaged and currents from individual KChAP-transfected cells were normalized to average control current. Those ratios were then averaged and plotted as fraction of control current. *Significant increase in current amplitude in KChAP-transfected cells. HERG tail currents were recorded at test potentials to −120 mV with a prepulse to +40 mV. KvLQT1 + minK currents were recorded at end of a 2.5-s pulse to +60 mV. For all transfected cells, recordings were made 2 days after transfection.

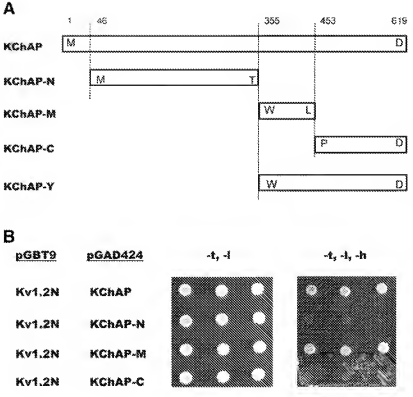

Fig. 5. Yeast two-hybrid assay of interaction of KChAP fragments with Kv1.2 amino terminus. *A*: schematic diagram of KChAP fragments. *B*: yeast two-hybrid interaction assay. Yeast host strain *HF7c* was cotransformed with GAL4-binding domain (pGBT9) and activation domain (pGAD424) fusion plasmids as indicated, and initially plated on media lacking tryptophan and leucine (−t, −l). Three individual colonies from each cotransformation were respotted on media with (−t, −l) and without histidine (−t, −l, −h) to follow activation of *HIS3* reporter gene as shown on *right*. Growth on media without histidine is indicative of an interaction. All constructs were negative for autonomous activation of transcription before use in this assay (not shown).

Kv1.2-N as evidenced by activation of the *HIS3* reporter gene and growth on minus histidine media (Fig. 5*B*). Neither KChAP-N, an amino-terminal KChAP fragment consisting of residues 46–354, nor KChAP-C (the carboxy-terminal portion of the protein from residues 453 to 619) gave a positive result in the yeast two-hybrid assay. Similar results were obtained when the panel of fragments was tested for interaction with Kv2.1-N and Kvβ1.2 (not shown). The assignment of the Kvα and β binding region of KChAP to this stretch of 98 residues is consistent with the KChAP fragment that was initially isolated in the yeast two-hybrid screen, KChAP-Y (30). KChAP-Y consisted of W355 through D619 (Fig. 5*A*).

KChAP and KChAP-M Increase Kv4.3 Current Amplitude and Total Protein in L Cells

In *Xenopus* oocytes, increased functional expression of Kv2.1 with KChAP was reflected in an increase in the total amount of Kv2.1 protein and the number of functional Kv2.1 channels (30). In the same experiments, we found that, 48 h after injection of KChAP cRNA in oocytes, KChAP protein was present in the nucleus and the soluble and membrane fractions. Kv2.1 protein was visualized at the cell surface but KChAP was not, suggesting that KChAP and Kv2.1 interacted transiently at some point before the channel reaches the cell surface. The evidence of increased Kv2.1 current, increased Kv2.1 protein, and direct but transient interactions between Kv2.1 and KChAP led to our hypothesis that KChAP is a chaperone for Kv2.1 in *Xenopus* oocytes.

To determine whether KChAP behaved similarly in mammalian cells, we examined L*Kv4.3* cells that were transfected with EGFP-KChAP with an anti-Kv4.3 polyclonal antibody and immunofluorescence microscopy. For comparison, cells from the same transfection experiment were recorded and electrophysiological data compiled. L*Kv4.3* cells transfected with EGFP-KChAP are shown in Fig. 6*A*. Fluorescence from EGFP-KChAP was observed in the nuclei of transiently transfected cells (Fig. 6, *A* and *B*, *right*). When we stained EGFP-KChAP transfected cells with a polyclonal antibody raised against a peptide in the carboxy terminus of KChAP (anti-KChAP-CTpep), we observed both cytoplasmic and nuclear staining in green fluorescing cells (Fig. 6*B*, *right*). Cells expressing EGFP-KChAP exhibited dramatically increased staining with the Kv4.3 antibody compared with nontransiently transfected L*Kv4.3* cells (Fig. 6*A*, *left*). Kv4.3 staining was not restricted to the cell surface but was especially bright in the perinuclear region, consistent with endoplasmic reticulum staining. Thus KChAP increased Kv4.3 total protein in L cells as it did for Kv2.1 in *Xenopus* oocytes.

Having localized the Kvα binding domain of KChAP to residues 355–452 (KChAP-M), we wanted to determine whether this fragment would also increase Kv4.3 protein in this assay. L*Kv4.3* cells were transiently transfected with EGFP-tagged KChAP-M and the cells stained with anti-Kv4.3 antibody. KChAP-M appeared to be in both the nucleus and cytoplasm, especially in the perinuclear region (Fig. 6*C*, *left*). In the same cell, we saw a dramatic increase in anti-Kv4.3 reactivity comparable to what was observed with full-length

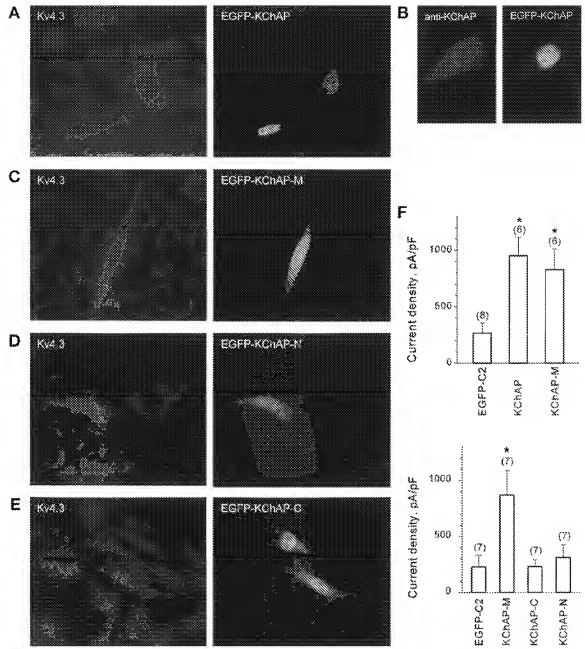

Fig. 6. KChAP and KChAP-M both increase Kv4.3 protein and current levels in mammalian cells. The stable cell line, L.Kv4.3, was transiently transfected with the following EGFP-tagged constructs: KChAP (*A* and *B*), KChAP-M (*C*), KChAP-N (*D*), KChAP-C (*E*). Cells were fixed 48 h after transfection, permeabilized, and stained with primary antibodies to either Kv4.3 (polyclonal; Alomone Labs; 1:200 dilution; *A, C, D,* and *E*) or KChAP (polyclonal KChAP-CTpep; 1:100 dilution; *B*) followed by a tetramethylrhodamine B isothiocyanate-conjugated anti-rabbit secondary antibody. *Right panels*: localization of EGFP-tagged proteins; *left panels*: costaining of Kv4.3 antibody (*A, C, D, E*) or KChAP (*B*) in same cells. Because this is a stable cell line, all cells exhibit a weak background staining with Kv4.3. Those that are transfected with KChAP or KChAP-M show increased staining with the Kv4.3 antibody. Note that, although the fluorescence of EGFP-tagged KChAP appears to be restricted to the nucleus, the KChAP antibody reveals cytoplasmic staining in transfected cells as well. *F*: K^+ current density in L.Kv4.3 cells transiently transfected with EGFP-tagged constructs. Currents were recorded 48 h post transfection. Note that both KChAP and the Kv channel binding fragment, KChAP-M, increase Kv4.3 currents, whereas KChAP-N and KChAP-C do not. *Values significantly different from cells transfected with EGFP-C2 alone. Numbers above bars refer to the number of cells of each type recorded.

KChAP (Fig. 6*C*, *right*). Figure 6*F* presents a summary of the K^+ currents that were recorded from transiently transfected L.Kv4.3 cells. Both KChAP and KChAP-M produced increased currents of comparable magnitude relative to EGFP-C2 alone. We also examined the effects of EGFP-tagged fusions of KChAP-N and KChAP-C on Kv4.3 protein and current levels. KChAP-N (Fig. 6*D*, *right*) showed a diffuse cytoplasmic localization with no concomitant increase in Kv4.3 staining (Fig. 6*D*, *left*). KChAP-C (Fig. 6*E*, *right*) ap-

peared to be present in both the nucleus and cytoplasm, but again produced no increase in the amount of Kv4.3 protein (Fig. 6*E*, *right*). The lack of increased Kv4.3 immunoreactivity with these two fragments was reflected in the lack of increase in Kv4.3 current density in cells overexpressing them as well (Fig. 6*F*). Thus the small fragment of KChAP that was identified as binding to Kvα amino termini was sufficient to produce increases in Kv4.3 in mammalian cells.

Transcription Is Not Required for KChAP Modulation of Kv Channels in Xenopus Oocytes

The partial nuclear localization of overexpressed KChAP in both oocytes and mammalian cells raises the question of whether KChAP is affecting the transcription of a gene that may influence the activity of Kv channels in our experiments. This is particularly relevant since KChAP-related proteins have been identified as functioning as transcription factor regulatory proteins (6, 19, 21, 28, 31). Despite our evidence that KChAP and certain Kv channels interact directly (30), we had to consider the possibility that nuclear KChAP was modulating Kv channels indirectly through a mechanism that involved transcription. We could test this possibility in oocyte experiments by examining KChAP effects on Kv channels in oocytes incubated with actinomycin D, an inhibitor of transcription. Figure 7 shows that KChAP increased Kv1.3 currents, irrespective of whether the oocytes were incubated with actinomycin D after injection or not (Fig. 7*A*). To confirm that actinomycin D could be effective, we injected Kv1.3 cDNA into oocyte nuclei and saw that, as expected, actinomycin D significantly reduced Kv1.3 currents in comparison to control (Fig. 7*B*). We observed similar results with Kv2.1 cRNA, cDNA, and KChAP (not shown). Thus transcription is not required for KChAP to increase Kv channel amplitudes.

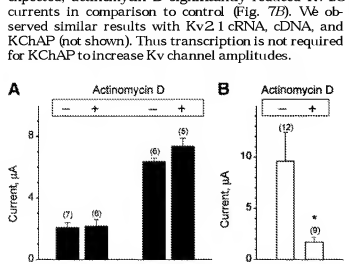

Fig. 7. Transcription inhibitor actinomycin D does not alter KChAP effects on functional expression of Kv1.3 in *Xenopus* oocytes. *Xenopus* oocytes were injected with cRNAs (*A*): Kv1.3 (0.5 ng/μl) alone or + KChAP cRNA (12.5 ng/μl); or with Kv1.3 cDNA (1.6 ng/μl; *B*). After injection, oocytes were divided into 2 groups and incubated for 24 h in media with or without actinomycin D (50 μg/ml). Currents were measured as described above (see Fig. 1). *Significant suppression of Kv1.3 current by actinomycin D in oocytes injected with Kv1.3 cDNA. Similar results were obtained on 4 batches of oocytes with Kv1.3

Association of KChAP With Kv2.1 and Kv4.3 in Rat Heart

To assess the physiological relevance of KChAP interactions with Kv channels, we raised a polyclonal antisera to KChAP and used it to probe for association with Kv channels in native tissue. The anti-KChAP antibody was raised against a KChAP fragment consisting of residues 453–619, was affinity purified, and was initially tested for its ability to detect overexpressed KChAP in *Xenopus* oocytes. A single band of ~68 kDa was detected in lysates of oocytes injected with KChAP cRNA, consistent with the predicted molecular mass of KChAP (Fig. 8*A*). In lysates from adult rat heart, the anti-KChAP antibody detected a band >75 kDa. This suggests that KChAP may be posttranslationally modified in heart, leading to an increased apparent molecular mass.

Because Kv2.1 and Kv4.3 are both sensitive to the effects of KChAP in heterologous expression assays and are expressed in adult rat heart, we searched for complexes of KChAP and Kv channel proteins by coimmunoprecipitation with anti-KChAP. Adult rat heart lysate immunoblotted with anti-Kv2.1 revealed two bands of ~105 and 130 kDa (Fig. 8*C*). These observations are consistent with the Western blotting pattern of Kv2.1 in rat heart (32) and brain (25). The 105-kDa Kv2.1 band coimmunoprecipitated with KChAP as shown in Fig. 8*C*. Interestingly, the larger 130-kDa Kv2.1 band, which is thought to be hyperphosphorylated (23), was not detected in KChAP immunoprecipitates. A polyclonal antibody to Kv4.3 detected a single band of ~75 kDa in adult rat heart lysates, which was present in complexes coimmunoprecipitated with the KChAP antibody (Fig. 8*D*). These data indicate that KChAP interacts with Kv channels in native tissue.

DISCUSSION

Our results demonstrate that KChAP modulates the functional expression of specific Kv channels without changing channel properties such as gating or voltage dependence in both *Xenopus* oocyte and mammalian expression systems. Following our initial characterization of KChAP and Kv2.1, we hypothesized that KChAP was a novel chaperone that interacted transiently with the channel but did not remain attached to the mature channel complex at the cell surface (30). Considered together with our previous data, the results reported here support the view that interaction of KChAP with Kv channels is responsible for the observed increase in current and protein levels. First, the coimmunoprecipitation of complexes of KChAP with Kv2.1 and Kv4.3 from rat heart lysates demonstrates that the proteins do interact in vivo. Second, yeast two-hybrid data suggest a direct interaction between KChAP and the amino termini of Kv1 and Kv2 channels as we had reported previously (30) but confirmed here for Kv1.3 N. Kv3 and Kv4 amino termini produced nonspecific transactivation of reporter genes in yeast and could not be assayed. In previous oocyte experiments, we only ob-

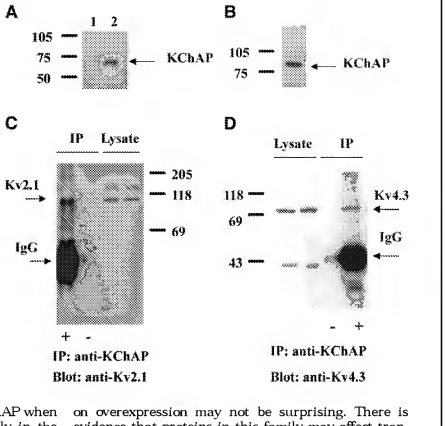

Fig. 8. Coimmunoprecipitation of KChAP with Kv channels from native tissue. *A*: Western blot of lysates (10 μg protein loaded per lane) from uninjected oocytes (*lane 1*) or oocytes injected with KChAP cRNA (*lane 2*) using affinity-purified KChAP polyclonal antibody (1: 100 dilution). *B*: Western blot of adult rat heart lysate (40 μg protein loaded) with affinity-purified KChAP antibody as in *A*. *C* and *D*: adult rat heart lysates were incubated with (+) or without (−) KChAP antibody (1:100 dilution). Immunoprecipitates (IP) were collected on Dynabeads and presence of Kv channels probed by Western blotting. *C*: Western blot with anti-Kv2.1 polyclonal antibody (Upstate Biotech; 1:200 dilution). *D*: Western blot with anti-Kv4.3 polyclonal antibody (Alomone Labs; 1:150 dilution). Lysate (40 μg protein loaded) and IP (− and + anti-KChAP) are shown.

served increases in Kv2.1 currents with KChAP when the two cRNAs were injected simultaneously in the same pipette, suggesting a requirement for the cotranslational association of the two proteins, and saw that a partial amino-terminal deletion of Kv2.1, although producing functional channels, was not sensitive to KChAP (30).

The evidence that KChAP is not part of the mature channel complex at the cell surface comes from heterologous expression experiments. In transiently transfected mammalian cells we have observed EGFP-tagged KChAP in both the nucleus and cytoplasm of the cell when examined 48 h after transfection. Interestingly, however, a partial fragment of KChAP, KChAP-M (98 residues), which retained the ability to bind to Kv channels, gave a punctate appearance in the cytoplasm reminiscent of Kv channel staining, suggesting a possible extended interaction of the two proteins.

KChAP belongs to a newly described multigene family consisting of PIAS1 (6), GBP (28) or PIAS γ (19), and Miz 1 (31) or ARIP3 (21). KChAP is most homologous to PIAS3, and examination of the nucleotide sequences suggests that the two may be the products of alternative splicing of a single gene. The difference between the two proteins is a 35-amino acid insert in the amino-terminal region of KChAP, which is not present in PIAS3. The Kv channel binding region, KChAP-M, is present in both KChAP and PIAS3, so it is likely that both KChAP and PIAS3 will interact with channels. All of the proteins in this family were cloned in yeast two-hybrid screens as binding to proteins that at least partly reside in the nucleus. PIAS3 was cloned as a STAT3-binding protein and is thought to function as an inactivator of activated transcription factor, STAT3. Thus the partial localization of KChAP to the nucleus on overexpression may not be surprising. There is evidence that proteins in this family may affect transcription either directly or indirectly. Our results suggest, however, that the effect of KChAP on Kv channels appears to be independent of events that occur in the nucleus. Blocking transcription in *Xenopus* oocytes with actinomycin D did not alter the ability of KChAP to increase Kv1.3 and Kv2.1 currents. Thus, even though KChAP may serve additional roles in the nucleus, our data are consistent with its effects on ion channels occurring through a transient interaction with the channel protein.

The actions of KChAP differ from the other family of Kv channel modulatory proteins, Kvβ-subunits. While Kvβ-subunits have also been described as having chaperone-like effects in that they increase the functional expression of some Kv1 channels, they remain tightly attached to the channels at the cell surface as with other hetero-oligomeric channels such as the ACh receptor (17, 18) and Ca^{2+} channel (3, 12). These hetero-oligomers are in marked contrast to KChAP, for which we have no evidence that KChAP:Kv channel complexes exist at the cell surface. Kvβ-subunits also bind to KChAP, however. Our data suggest that KChAP and Kvβ-subunits may compete for binding to Kvα1 amino termini, producing alterations in current properties (unpublished observations).

Our data show that a restricted subgroup of Kv channels is sensitive to modulation by KChAP: Kv1.3, Kv2.1, Kv2.2, and Kv4.3 (data presented here and in Ref. 30). It is not clear at present why some Kv channels are sensitive to modulation by KChAP and others are not. Among the Kv1 subfamily, only Kv1.3 currents increase on coexpression with KChAP, even

though the amino termini of other family members, such as Kv 1.2, also bind to KChAP in yeast two-hybrid assays. Preliminary experiments with chimeric channels of Kv 1.2 and Kv 1.3 suggest that neither the amino nor carboxy termini of Kv 1.3 confer sensitivity to KChAP and point to the core domain as critical.

Given its homology with PIAS3, it is intriguing to speculate that KChAP may function as an integral component of a signaling pathway linking nuclear processes with the expression of ion channels at the cell surface. Perhaps by regulating KChAP binding to Kv channels, the expression of Kv currents at the cell surface can be modulated in a rapid, acute manner, independent of changes in Kv channel gene transcription. Whether KChAP is available for binding to Kv channels could depend on its binding to other targets such as STAT3. Further experiments are required to sort out the meaning of these possible multiple interactions in vivo.

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